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FOREWORD

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Introduction

Nature of the Problem

Defects in cell-cell adhesion are commonly associated with tumor progression. There is evidence that alterations in the expression of the calcium-dependent cell adhesion molecule E-cadherin occur in a subset of invasive breast cancers and breast cancer cell lines. However, many invasive breast cancers and metastases are E-cadherin positive. Preliminary results indicate that breast tumor progression may more often be accompanied by alterations in the expression and function of several cadherin-associated molecules that are essential for cadherin-mediated cell-cell adhesion. It is the aim of this proposal to test the hypothesis that, in addition to the occasional loss of E-cadherin expression, breast tumor progression is more realistically modeled by a defect in cell-cell adhesion resulting from an alteration in any one or more of the steps (molecules) required for E-cadherin function. We will take two fundamental approaches. Firstly, we will use two methods for "non-specifically" assessing E-cadherin function and cell-cell adhesive strength in breast tumor samples and cell lines. Secondly, we will specifically investigate the molecular mechanisms that lead to defects in cell-cell adhesion by examining (and manipulating) the expression and phosphorylation state of several E-cadherin associated molecules in breast tumors and cell lines.

Background and Previous Work

Cell Adhesion, Cell junctions and Cancer The concept that alterations in cell to cell adhesion and intercellular communication are involved in tumorigenesis and tumor progression is certainly not new (1). However, it is only recently that the molecular basis underlying these changes has begun to be addressed. Homotypic cell-cell adhesion molecules (CAMs) and cell-cell junction molecules have now been implicated as tumor and metastasis suppressor genes in several systems (2-8). A gene often deleted in colon carcinoma (DCC) and associated with colon tumor progression is likely to be a homotypic CAM of the immunoglobulin superfamily (9). Of most interest to the present proposal are the calcium-dependent class of CAMs, cadherins, characteristically expressed by cells of epithelial origin (10,11). Cadherin-mediated adhesion is fundamentally involved in the organization of epithelial tissues during development, and manipulations of cadherin function result in profound disturbances of tissue organization (10-13). Several drosophila tumor suppressor genes are either cadherins or junction associated molecules and reduction in cadherin expression has been associated with the malignant phenotype in many advanced human carcinomas (2-8). In order for cadherins to function in cell-cell adhesion and promote the formation of junctions several other associated molecules need to be expressed (11,14-16). These molecules known as catenins, link the cadherins to the underlying actin cytoskeleton and are probably involved in propagating adhesion-related signalling (16,17). Our preliminary results show that the expression of certain catenins is lost in malignant breast carcinoma cells (16). Other studies have indicated that the phosphorylation state of catenins can also influence the transformed phenotype (17). The mechanism whereby alterations in cadherin-mediated adhesion affects cell proliferation, morphological differentiation and invasion is unknown. All differentiated epithelial cell collectives are linked by gap junctions permeable to intracellular calcium. In this situation, changes in intracellular calcium can be propagated rapidly among communicating cells. It is therefore not surprising that many tumor cells including breast tumor cells are deficient in gap junctional communication ((18) and references therein).

Interestingly, transfection of E-cadherin into squamous cell carcinoma cells lacking gap junction function results in the expression of the gap junction proteins connexins and the assembly of functional gap junctions (19).

E-cadherin in Breast Cancer The presence of lymph node and distant metastases predict poor prognosis for cancer patients. For example, in a large clinical trial, percent treatment failure at 5 years for patients with no lymph node involvement upon histological examination was 13%, for those with 1-3 positive nodes the failure rate was 39% and for those with >4 positive nodes the failure rate was 69% (reviewed in (20)). These results underscore the necessity for research designed to understand the process of metastasis and to discover molecular markers that will predict whether a given tumor is likely to metastasize. The study of E-cadherin and associated protein expression and function in breast cancer cells can potentially provide information pertinent to both of these aims.

Several studies have examined the expression of E-cadherin in human breast cancer tissues (21-23). Loss or reduction of E-cadherin immunostaining was observed in a proportion of samples in each study. Normal breast epithelial structures consistently stain at cell-cell borders for E-cadherin. In one study (22), 53% of 120 tumors had reduced E-cadherin expression (defined as >10% of cells being E-cadherin-negative). The majority of the samples examined in this study were invasive ductal carcinomas, the most common form of breast cancer diagnosed. Loss of E-cadherin expression correlated with poorer differentiation state and with higher stage (T, N and M). In particular, 86% of samples from patients with distant metastasis (M₁) had reduced E-cadherin staining whereas 47% of samples from patients with no known distant metastasis (M₀) had reduced E-cadherin expression (22). Similar results were reported in a smaller study by Gamallo et al. (21). A third study examined a larger number of invasive lobular carcinomas (23). Complete loss of E-cadherin expression was detected in 29 of 35 samples. The remaining 6 samples had a diffuse staining pattern for E-cadherin. The ductal carcinoma samples had variable intensities of staining for E-cadherin although the proportions of E-cadherin-negative cells in each sample was not quantitated. Taken together, these results indicate that E-cadherin expression is lost in a significant proportion of lobular carcinoma specimens and reduced E-cadherin expression correlated with higher stage (poorer prognosis) and poorer differentiation in invasive ductal carcinomas.

One difficulty in comparing the results of E-cadherin staining of tumor tissues is the inconsistency between observers regarding when to classify a tumor as "E-cadherin-positive" or "E-cadherin-negative". In the study by Oka et al. (22), tumors with >10% of cells displaying no E-cadherin immunostaining were classified as having "reduced expression". However, in many studies no such cutoff was used (e.g. (21)). Many studies also describe "diffuse" or "disorganized" or "reduced" staining patterns for E-cadherin (21,23,24). These descriptions may indicate a defect in connection of E-cadherin to the actin cytoskeleton in these samples which could lead to a loss of adhesive function even in the presence of immunoreactive E-cadherin.

Although a trend between increasing stage and reduced E-cadherin expression was observed in breast cancer (22), the ability to predict metastatic spread based on expression of E-cadherin in a primary breast tumor is uncertain. An analysis of E-cadherin expression in 19 lymph node metastases and in their primary tumors was performed (22). Of the primary tumors, six were E-cadherin-positive, five were E-cadherin-negative and eight had a mixed phenotype. Five of the

six lymph node metastases from E-cadherin-positive primary tumors were E-cadherin-positive and one was mixed. All five of the lymph node metastases from E-cadherin-negative primary tumors were E-cadherin-negative. Among the lymph node metastases from the eight mixed primary tumors, three were E-cadherin-positive, two were E-cadherin-negative and two were mixed. The fact that five of the eight lymph node metastases from mixed primary tumors contained E-cadherin-positive indicates that a selection for E-cadherin-negative cells in the metastatic process does not occur.

Rationale and Hypothesis to be Tested The discussion above together with our preliminary data has led us to conclude that E-cadherin expression alone is a poor predictor of breast tumor invasive potential and metastatic spread. Significant loss of heterozygosity (LOH) of chromosomal locus 16q occurs in several carcinomas including breast (25-27). The human E-cadherin gene is localized to 16q22.1 and it is possible that an apparent reduction in staining intensity in some tumors may be due to this LOH. In breast cancer 16q LOH is correlated with distant metastatic spread. Although loss of functional homotypic cell-cell adhesion and intercellular communication are clearly associated with the transformed malignant epithelial cell phenotype this is not necessarily due to loss of cadherin expression or LOH at the cadherin locus. A defect in any one of the molecules involved in cadherin function, or a change in any of the pathways involved in cadherin responsive intra- and inter-cellular signalling could also result in the same phenotype. Other cell-cell adhesion molecules not discussed here are also likely to be affected. In other words, the two important carcinoma cell adhesion related phenotypes of 1) alterations in contact dependent growth and 2) invasion and metastasis, can be achieved in many different ways. Based on our own preliminary results and those in the literature we have calculated that there are several hundred potential routes whereby functional cell-cell adhesion could be altered during carcinogenesis and result in these phenotypes. Bearing in mind that we are limited by current knowledge this number is likely to be conservative. Not surprisingly, more than a dozen lesions in the cadherin-related adhesion system alone have already been uncovered in various carcinomas and cell lines (see discussion above). Whilst it is clearly of great importance to continue cataloging these molecular changes, indeed we propose to do so in one of our specific aims, it is equally important to develop methods in which functional cell-cell adhesion can be assessed directly. Such methods should uncover any defect in cell-cell adhesive strength no matter what the underlying molecular basis.

Body

Experimental Design And Methods

Task 1. To test the hypothesis that cell-cell adhesive strength and E-cadherin triton solubility is correlated with functional E-cadherin-mediated cell-cell adhesion. Years 1-4

The first "non-specific" approach that we will use for assessing tumor cell-cell adhesion strength is based on our recently described laminar flow assay (28). Using this assay developed originally to investigate cell-substratum interactions, we hypothesized that we should be able to distinguish cell-cell adhesive strength among tumor cells which express functional and non-functional E-cadherin. We will extend these studies to many more cell lines and to tumors derived from these lines. We will refine our procedures using tumors derived from cells which we already know express functional and non-functional E-cadherin and which are of known adhesive strength *in vitro*. It is not the goal of this specific aim to use these direct assays of cell-cell adhesive strength

as a routine screening procedure for breast cancer. For each sample we will correlate cell-cell adhesion strength with E-cadherin triton solubility. We will restrict our analyses to tumors in which E-cadherin is present (but perhaps non-functional). In this way we can be certain that the cell aggregates that we analyze are derived from the tumor itself rather than any stromal elements which may contaminate it, since these will be E-cadherin negative and will not exhibit calcium-dependent cell-cell adhesion. We are not so interested in E-cadherin negative tumors since these have an obviously demonstrable lesion in cell-cell adhesion.

Task 1. Methods and Results (refer previous report and to figures in appendix 1)

Most of the experiments which we proposed to carry out in aim 1 have been completed (see previous report). Some of this work has now been published and some has been submitted for publication (see appendix 1). Although not proposed in the original grant we have also used the adhesion strength assays in an embryonal carcinoma cell system to demonstrate for the first time that the actin binding protein vinculin (similar to α -catenin) is required for the generation of high cell-cell adhesion strength (see appendix 1). The remaining aspects of task 1 involve the application of these assay systems to cells which have been isolated from tumors growing in nude mice. These studies are underway but we do not have results yet.

We will first refine our procedures using tumors derived from cells which we already know express functional and non-functional E-cadherin and which are of known adhesive strength *in vitro*. It is not the goal of this specific aim to use these direct assays of cell-cell adhesive strength as a routine screening procedure for breast cancer. For each sample we will correlate cell-cell adhesion strength with E-cadherin triton solubility (see below). We will restrict our analyses to tumors in which E-cadherin is present (but perhaps non-functional). In this way we can be certain that the cell aggregates that we analyze are derived from the tumor itself rather than any stromal elements which may contaminate it, since these will be E-cadherin negative and will not exhibit calcium-dependent cell-cell adhesion. We are not so interested in E-cadherin negative tumors since these have an obviously demonstrable lesion in cell-cell adhesion.

Nude mice tumors In previous studies we have generated tumors in nude mice from several breast carcinoma cell lines. Some of these have been transfected with E-cadherin. We will continue to do this in the present proposal. 5 million carcinoma cells of varying E-cadherin status will be injected into the right upper mammary fat pad of 4-6 week-old athymic female nude mice (BALB/c-*nu/nu*) and the injection sites observed once or twice a week for the appearance and size of primary tumors. For estrogen responsive cells, 17-beta estradiol pellets (0.72 mg, 60 day release, Innovative Research of America) will be implanted in the interscapular region. After 3-6 weeks mice are sacrificed and tumor tissue used for the preparation of cells and for frozen sections. Cells from the tumors will be analyzed using the laminar flow assay and cell-cell adhesion strength calculated as we described for the cell lines (see previous report and (28))

Task 2. To measure the expression and phosphorylation state of cadherin-associated proteins in breast tumors and cell lines (Years 1-4). We will examine the expression and phosphorylation state of the cadherin-associated proteins alpha catenin, beta catenin and plakoglobin in breast tumors and cell lines.

Task 2. Methods and Results

The methods for immunoprecipitation and western blot analysis were described in the previous report. At that time we had shown that serine rather than tyrosine phosphorylation was the prominent post-translational modification of β -catenin in breast cancer cells (see previous report). During the present grant period we extended this work to demonstrate that serine phosphorylation of β -catenin has profound effects on its stability and cellular localization. We have found that β -catenin in breast cancer cells is targeted for ubiquitination and proteasomal degradation by phosphorylation of a specific serine residue at the N-terminal (see figure 1 for a summary). Our results also indicate that the product of the tumor suppressor gene APC is involved in this pathway. In addition, in those cells in which β -catenin protein levels are low, mRNA levels are unaffected indicating that regulation of β -catenin expression occurs predominantly at the protein level (see (29)). This regulation of β -catenin protein levels by serine phosphorylation may well have implications in breast cancer since β -catenin is a central element in the wnt-1 signaling pathway (30). As emphasized in the original proposal wnt-1 overexpression is known to cause breast cancer in mice (31). □

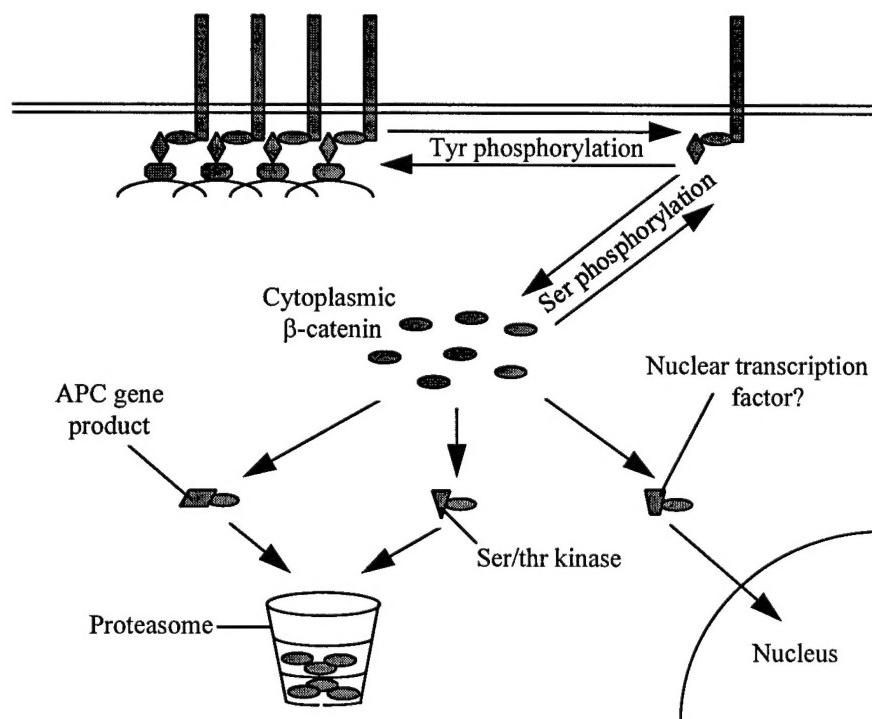


Figure 1. Hypothesized Regulation of cadherin and β -catenin function. Tyrosine phosphorylation of β -catenin inhibits cadherin-mediated adhesion and moves cadherin/catenin complexes out of the insoluble, cytoskeleton-linked fraction. Preliminary results indicate that movement of β -catenin between cytoplasmic and membrane-associated pools is regulated by ser/thr kinase activity, however, the target of that activity is unknown. The cytoplasmic level of β -catenin is regulated by protein stability. It has

been hypothesized that ser/thr phosphorylation and interactions between APC and β -catenin may target β -catenin for destruction by the proteasome. Decreased cytoplasmic β -catenin levels inhibits its signaling function. Yeast and human receptors for nuclear localization sequences are also members of the armadillo family indicating that cytoplasmic β -catenin may act to either chaperone other molecules (e.g. transcription factors) to the nucleus, or act to retain them in the cytoplasm.

Task 3. Statistical analyses (years 3-4). Results will be correlated with tumor stage, blood vessel count, lymph node status, the expression of prognostic markers and period of metastasis-free survival. We are presently accumulating data on phosphorylation status and detergent solubility of cadherins and catenins in breast tumor tissues. This will continue until year 4 when we should have enough material to carry out a statistical analysis

Task 4. To directly examine the role of phosphorylation and plakoglobin expression on breast cancer cell-cell adhesion strength (Years 1-4). We will examine the role of phosphorylation and plakoglobin expression on breast cancer cell-cell adhesion strength by directly examining the effects of kinase inhibitors and plakoglobin transfection on cell-cell adhesion strength using biophysical methods.

Task 4. Methods and Results

To directly examine the role of phosphorylation and plakoglobin expression on breast cancer cell-cell adhesion. In the previous report we presented preliminary results showing that in two invasive cell lines β -catenin is constitutively heavily tyrosine phosphorylated. During the present grant period we went on to test the hypothesis that this hyperphosphorylation is the cause of the failure of transfected E-cadherin to alter the phenotype of the cells. In pilot experiments we found that only one of the cell lines (BT549) responds to tyrosine kinase inhibitors by alterations in E-cadherin-mediated adhesion. We have now used several classes of kinase inhibitors the most effective being herbimycin A. We continued these experiments and ascertained that herbimycin A did not alter the response of the cells in terms of adhesion strength, but did result in the restriction of E-cadherin to cell-cell contact sites. The second cell line (HS578T) did not respond to any of the tyrosine kinase inhibitors even at very high doses. Unlike BT549 cells which express low but detectable levels of the cadherin-associated molecule plakoglobin, HS578T cells do not express plakoglobin (32). We intended to determine whether the lack of plakoglobin is responsible for the failure of HS578T cells to respond to E-cadherin transfection by expressing plakoglobin cDNA in the cells. Although we were successful in generating cell lines which expressed plakoglobin we found that increased plakoglobin expression did not make the cells more adhesive even in the presence of E-cadherin.

Invasive E-cadherin negative breast cancer cells express high levels of the mesenchymal cadherin, cadherin 11

Our failure to reverse the poorly adhesive phenotype of invasive breast cancer cells by exogenous expression of E-cadherin or plakoglobin or by inhibition of tyrosine kinase activity prompted us to re-examine the cadherin profile of these cells. In our earlier studies we had generated a model in which invasive breast cancer cells had resulted from an epithelial to mesenchymal transition (EMT (33,34)). In contrast to general opinion, recent data in developing systems has demonstrated that during EMTs the resulting mesenchymal cells do in fact express a cadherin, now characterized as cadherin 11(35). We had demonstrated previously that the invasive breast cancer cells did exhibit a weak form of calcium-dependent adhesion even though they did not express E-cadherin. Immunoprecipitation studies with antibodies to β -catenin also showed that a band of the appropriate molecular weight for a cadherin (~120 kD) was co-precipitated. Western blots with a pan-cadherin antibody also demonstrated the presence of high levels of an unknown cadherin in these cells. We recently investigated the expression of cadherin 11 in breast cancer cells by RT-PCR and our results are summarized in table 1. Cadherin 11 is expressed in all the invasive breast cancer cells and is never expressed with E-cadherin. This rather dramatic finding adds fuel to our argument that breast cancer progression may well be in part due to an EMT.

Table 1. Relationship among morphology/invasive potential and E-cadherin and cadherin 11 expression. Note that only stellate/invasive cell lines express cadherin 11 and the inverse relationship between the two cadherins. Transfection of E-cadherin into cadherin 11 positive cells (BT-Ecad and HS-Ecad) does not decrease cadherin 11 expression or reverse the invasive phenotype.

| Cell Line | Morphology/invas. | E-cadherin | Cadherin 11 |
|-------------|-------------------|------------|-------------|
| A1N4 | Fused | + | - |
| MCF10A | Fused | + | - |
| MCF-7 | Fused | + | - |
| ZR-75-B | Fused | + | - |
| T47D | Fused | + | - |
| BT474 | Fused | + | - |
| MDA-MB-468 | Fused/spherical | +/- | - |
| SKBR3 | Spherical | - | - |
| CAMA-1 | Spherical | - | - |
| MDA-MB-453 | Spherical | - | - |
| MDA-MB -134 | Spherical | - | - |
| HBL-100 | nd | - | + |
| MDA-MB-436 | Stellate | - | + |
| MDA-MB-435 | Stellate | - | + |
| MDA-MB-231 | Stellate | - | + |
| BT549 | Stellate | - | + |
| BT-E-cad | Stellate | + | + |
| HS578T | Stellate | - | + |
| HS-E-cad | Stellate | + | + |

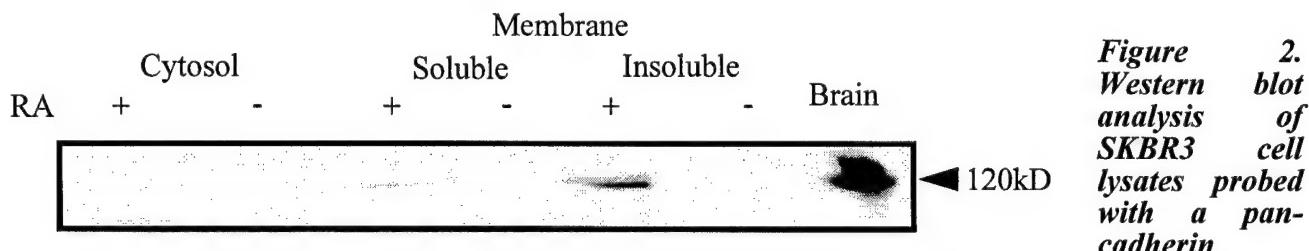
Recent studies in squamous carcinoma cells demonstrate that invasive cell lines express N-cadherin rather than E-cadherin and that specific blockade of N-cadherin reversed invasion and tumorigenicity and also allowed the cells to re-express E-cadherin (36). These data indicate that it is possible that invasive breast cancer cells express cadherin 11 which is in fact essential for their invasive phenotype. Perhaps specific blockade of cadherin 11 could reverse invasion and tumorigenicity. Another aspect of cadherin 11 is of some interest. Although cad 11 is expressed in embryonic mesenchyme it is not normally expressed in adult mesenchymal cells. However, it is expressed in osteoblasts raising the possibility that cad 11 expression by carcinoma cells could increase the opportunity of these cells to metastasize to bone (37). Other possible avenues of investigation include:

1. The use of cad 11 as a marker for invasive or potentially invasive breast cancer
2. Exogenous regulated expression of cadherin 11 antisense to demonstrate relationship to invasiveness, tumorigenicity and bone metastases.

Retinoids Increase Cell-Cell Adhesion Strength, β -Catenin Protein Stability and Localization to the Cell Membrane in a Breast Cancer Cell Line. A Role for Serine Kinase Activity. During the present funding period we continued our work related to the effect of retinoids on the molecular and cellular aspects of cell-cell adhesion. A paper recently published

describes this work which was summarized in the last report. During the present funding period we have continued these experiments and have the following additional results.

1. Retinoid treatment results in increased expression of a cadherin and its localization to a triton insoluble pool at the cell membrane (Fig. 2). The cadherin was detected using the pan-cadherin antibody described earlier and we are in the process of identifying it now. Preliminary results indicate that it is not one of the common cadherins (it is not cad 11) and we will develop a screen expression library with the pan-cadherin antibody to identify it.



antibody. Note that a positive reaction is observed only in the membrane preparations from RA-treated cells. More cadherin reactivity is observed in the detergent insoluble fraction indicating that the cadherin is linked to the cytoskeleton.

2. In our paper we showed some immunocytochemical studies in which β -catenin was localized in control and retinoic acid treated cells. As stated earlier β -catenin levels are low in control cells, however, immunocytochemical staining indicated that approximately 5% of cells exhibited bright cytoplasmic β -catenin staining (29). These images indicated to us that a defined subpopulation of cells had elevated cytoplasmic levels of β -catenin, the most likely reason for this being that the positive cells were at a particular stage of the cell cycle. During the present funding period we pursued this further and confirmed our results in other cell lines in which the cells had been synchronized (see Fig. 3). Although preliminary, these results indicate that cytoplasmic β -catenin levels are elevated in late G1/S and point to a potential role for β -catenin in regulation of the cell cycle. Presumably, treatment of cells with retinoids increases the movement of β -catenin from this cytoplasmic pool to a stable membrane and cadherin-associated pool.

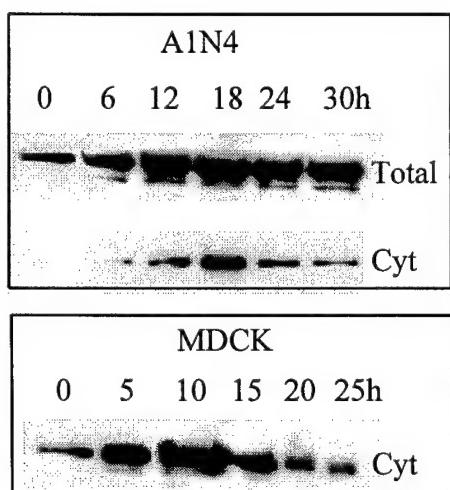


Figure 3. β -catenin protein levels vary as a function of the cell cycle in A1N4 and MDCK cells. Note that cytoplasmic but not total β -catenin levels oscillate during the cell cycle. The steady increase in total β -catenin levels is presumably a reflection of increased size and cell number as the cells pass through one cycle. Most A1N4 and MDCK cells have entered S-phase by 18 and 10h respectively as measured by FACS analysis of parallel plates.

Conclusions

Implication of the Completed Research

The major findings of the second year of work are:

- 1) Biophysical techniques were used to determine the role of vinculin in cell-cell adhesion strength. These results are significant since vinculin has been proposed to act as a tumor suppressor. In addition, this is the first study to directly show that vinculin has a defined function in the generation of high cell-cell adhesion strength as well as its known role in the formation of focal adhesions with the ECM.
- 2) We found that manipulations of tyrosine kinase activity did not increase cell-cell adhesion strength in E-cadherin negative breast cancer cells or in cells which expressed exogenous E-cadherin. Similarly, transfection of plakoglobin-deficient cells with plakoglobin did not increase adhesion strength.
- 3) We found that E-cadherin negative invasive cells all expressed the mesenchymal cadherin, cadherin 11.
- 4) Retinoic acid, acts on certain breast cancer cells to increase the expression of a novel cadherin and its localization to a detergent insoluble membrane fraction.
- 5) Serine phosphorylation of β -catenin targets it for ubiquitination and proteosomal degradation via a pathway involving the tumor suppressor gene APC.
- 6) Levels of β -catenin oscillate during the cell cycle implying a role in G1/S progression.

Recommended Changes

- 1) We will expand our studies to include investigations of cadherin 11.
- 2) We will identify the cadherin in SKBR3 cells

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Appendices

1. Tozeren,A., Wu,S., Hoxter,B., Adamson,E. and Byers, S.W. 1997 Laminar flow assays demonstrate the role of vinculin in cell substratum and cell-cell adhesion. Submitted
2. Byers, S., Pishvaian, M., Crockett, C., Peer, C., Tozeren, A., Sporn, M., Anzano, M., and Lechleider, R. Retinoids increase cell-cell adhesion strength, beta catenin protein stability, and localization to the cell membrane in a breast cancer cell line. A role for serine kinase activity. *Endocrinology*, 137: 3265-3273, 1996

**LAMINAR FLOW ASSAYS DEMONSTRATE A ROLE FOR VINCULIN
IN EMBRYONAL CARCINOMA CELL-CELL ADHESION**

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ABSTRACT

Vinculin is a constituent of adhesion plaques and is clearly involved in focal adhesion to the extracellular matrix. Vinculin is also found at cell-cell adherence junctions in non-muscle cells but no studies have addressed its role there. In this study the role of vinculin on the physical strength of cell-cell adhesion was studied directly by conducting disaggregation assays on aggregates of wild-type F9 mouse embryonal carcinoma cells, two vinculin-depleted F9 cell lines, and a reconstituted vinculin-depleted cell line that re-expresses vinculin. Both vinculin-positive and vinculin-negative F9 cells could form aggregates during a two-hour incubation at 37°C. However, aggregates of vinculin-depleted cells could be disaggregated with the imposition of simple shear flow ($>21 \text{ dyn/cm}^2$) whereas wild-type and vinculin-reconstituted cell aggregates remained intact in the presence of high levels of shear stress (77-91 dyn/cm^2). Since laminar flow imposed on cell aggregates acts to separate cells from each other, our data indicate that the loss of vinculin is associated with a significant reduction in the strength of cell-cell adhesion in newly formed aggregates. However, vinculin-dependent differences in cell-cell adhesion strength diminished when cell aggregates were formed over 48 hours. These results indicate that vinculin is not essential for the initial events in cell-cell adhesion, nor is it absolutely required for the formation of adherens junction of high adhesive strength, rather, it acts to modulate the rate of transition between the two states.

INTRODUCTION

Vinculin is a cytoskeletal protein closely associated with cell matrix and cell-cell junctions which binds strongly to actin-binding proteins such as α -actinin and talin. The affinity of vinculin for actin depends strongly on its conformational state suggesting that it serves as a dynamic link between the cell cytoskeleton and cell surface adhesion molecules such as integrins and cadherins (8,9).

Vinculin expression is dramatically decreased in many highly-malignant and metastatic tumor cells and under some circumstances the tumorigenity of these cells can be suppressed by exogenous expression of vinculin cDNA (15,16). Similarly, an important role for vinculin in development is suggested by studies that showed regulation of vinculin expression during embryonic morphogenesis and during cell migration *in vivo* (13,21). Culture of cells on highly adhesive matrices induces vinculin gene expression, indicating an important role for vinculin in cell-matrix adhesion (4).

To elucidate the exact role of vinculin in cell adhesion and motility, stable clones with vinculin-null mutations were generated by disrupting the vinculin gene in F9 embryonal carcinoma and embryonal stem cell clones using homologous recombination (4). Vinculin-null cells displayed a rounder morphology and a reduced ability to adhere to plastic and fibronectin after short periods of static incubation. Consistent with the observation that maximal capacity for

migration correlates with the reversibility of cell-substratum adhesion, loss of vinculin expression resulted in a significant increase in cell motility (4,6).

Although vinculin is clearly present in epithelial cell-cell junctions its role in cell-cell adhesion is yet to be determined. In this study, a laminar flow assay procedure was used to investigate the role of vinculin on the capacity of cell aggregates to resist shearing forces. Our study showed that lack of vinculin results in a significant reduction in cell-cell adhesion strength, particularly at early stages of aggregate formation.

METHODS

Cells

F9 mouse embryonal carcinoma cells (Clone B1M, (7)), two vinculin null F9 cell lines, γ 227 and γ 229 (4)and vinculin re-expressing γ 229 cells (R3) that express vinculin at 83% of B1M levels were used in these studies. R3 cells were obtained by transfecting the γ 229 cells with an expression vector in which the β -actin promoter drives the expression of full-length vinculin cDNA. The vector and the resulting cell lines will be described in another paper (W.Xu and E.D. Adamson, in preparation).

Indirect Immunofluorescence

Trypsinized washed cells were seeded onto fibronectin (5mg/ml) and polysine-coated coverslips ($5\text{mg}/\text{cm}^2$) and allowed to grow for 1 to 2 days (4). In this time individual colonies

containing from 20 to 40 cells were obtained, with the aim of observing both cell-matrix and cell-cell interfaces within the colonies. The cells were fixed 8 min in 4% paraformaldehyde and permeabilized for 2 min in 0.1% Triton in phosphate-buffered saline. The procedure for double fluorescence staining of vinculin and F-actin was as described previously (4). The cells were viewed in a Zeiss LSM 410 laser scanning confocal microscope and images printed in a Sony UP/D7000 digital printer.

C. Immunoblotting

Cells in monolayers at 70% confluence in 100 mm dishes were washed removed with a rubber policeman and lysed in Laemmli sample buffer without 2-mercaptoethanol and bromophenol Blue (BPB). Lysates containing 5 mg/ml leupeptin, 5 mg/ml aprotinin and 1 mM PMSF protease inhibitors were placed in boiling water for 10 min, an aliquot was removed for measurement of optical density and equal amounts of protein were analyzed at three different concentrations (the two lowest levels are shown in Fig. 2). The samples were further heated after being adjusted to contain 2-mercaptoethanol and BPB, and loaded into individual lanes for 7.5% SDS-PAGE (12). After electrotransfer to PVDF (Immobilon, Milipore Corp., Bedford, MA) membranes, the blots were processed to reveal E-cadherin, using a rabbit anti-mouse gp84 (19) at 10 µg/ml together with a mouse monoclonal antibody to β-actin (clone AC-15, Sigma Chemical). Suitable peroxidase secondary antibodies were used to reveal the protein on the membrane. The enhanced chemical luminescence procedure (ECL, Amersham, Little Chalfont, UK) was used to detect the antigen to which the primary and secondary antibodies had adsorbed. The resulting

protein signal on film was analyzed by NIH-Image to estimate the relative levels of each protein band. After quenching the first signal from the membrane, antibodies to α -catenin (C2081, Sigma) and β -catenin (C2206, Sigma) were used in a second cycle on the same membrane (11). The level of actin was used to normalize the levels of the other cytoskeletal proteins in each of the four cell lines.

D. Formation of Cell Aggregates

In order to obtain pre-formed cell aggregates, cells were harvested from subconfluent flasks of cells by vigorous pipetting. The resulting aggregates were then kept at room temperature (24°C) up to an hour before use in laminar flow assays. In another set of experiments disaggregation assays were performed on aggregates formed from single cells during a two-hour incubation period. Cells were removed from the plate by a 20 minute treatment with trypsin and the resulting single cells were resuspended in DMEM supplemented by 10 % fetal calf serum at a concentration of 1×10^6 cells/ml (Hyclone Lab., Inc., Logan, UT). Light microscopy confirmed the absence of cell doublets or larger aggregates in the original suspension. The resulting cell suspension was maintained for 2 hrs at 37°C in a 5% CO₂ incubator to regenerate cell surface proteins and to form cell-cell contacts. The disaggregation experiments were performed at 24°C during the next hour. To assure that the differences in the capacity of various cell lines to resist flow-induced disaggregation did not depend on the small differences in the incubation time, the order in which the various cell types were used in laminar flow assays were systematically altered. The results obtained did not vary significantly within the time period of the experiments.

E. Flow Chamber and Laminar Flow Assays

A parallel plate flow chamber of uniform width was used in the laminar flow assays. The chamber consisted of: (1) a transparent top plate having appropriate openings for the delivery of the fluid into and out of the channel; (2) a 0.15 mm thick thin plastic gasket; (3) a grade no. 1 glass coverslip serving as the bottom plate of the channel (Fisher Scientific, Pittsburgh, PA); and (4) top and bottom stainless steel cover plates with observation slots (18). The glass coverslips used as bottom plates were coated with fibronectin (Becton Dickinson Labware, Bedford, MA). Briefly, proteins were diluted in serum-free culture medium (DMEM) from a stock-solution to reach a final concentration of 50 $\mu\text{g}/\text{ml}$ and placed on the centers of coverslips in the form of 50 ml drops. Such drops eventually coat an approximately circular region of 0.5 cm^2 in area, yielding a surface concentration of $5 \mu\text{g}/\text{cm}^2$ (18). The coverslips were then incubated at 24°C for two hours and were rinsed in distilled water three times before use in the experiments. The bottom plate, the gasket and the top plate were fastened between the cover plates. The entry port of the chamber was connected through a valve and teflon tubing to a syringe filled with suspending medium. The cell suspension was infused directly into a chamber from a small opening through the top plate.

The shear stress on the bottom plate of the chamber in the direction of flow, τ_b (dyn/cm^2), was evaluated using the following equation, assuming Poiseuille flow:

$$\tau_b = 6\mu Q/(h^2 w) \quad (1)$$

where μ (~ 0.01 dyn-s/cm 2) is the viscosity of the cell medium, Q (cm 3 /s) is the flow rate, h (0.012 cm) is the gap thickness of the channel and w (1 cm) is the width of the channel (18).

Flow-induced disaggregation of large aggregates was performed as described earlier (3). Experiments were initiated by placing the flow chamber on the stage of an inverted microscope (Diaphot, Nikon Inc., Garden City, NJ). The cell suspension (containing cell aggregates as well as single cells) were infused into the chamber and allowed to interact with the fibronectin-coated glass coverslip under static conditions for 15 minutes. A syringe pump (Harvard Apparatus, South Natick, MA) was then used to pump the cell medium into the chamber at specified flow rates. A 40x phase contrast objective and a videocamera system (DAGE-MTI) attached to the side port of the microscope were used to record the disaggregation events.

Aggregates whose largest dimension before the imposition of flow was 70 to 140 μ m were classified as large aggregates. These aggregates typically contained multiple layers of cells, with many cells adherent to neighboring cells but not to the planar substratum. A detachment event was said to occur when a cell or a cluster of cells detached from the remaining stationary aggregate in response to the imposed flow. The average number of detachments per aggregate and its standard deviation were determined by quantifying the number of detachment events per aggregate for at least 12 large cell aggregates of a given cell type.

RESULTS

Location of Vinculin at Cell Margins

We used double immunofluorescence analysis to confirm the lack of vinculin and the presence of F-actin in γ 227 and γ 229 cells. The actin filaments were slightly shorter in the γ 229 cells due to the smaller diameter of these rounded cells, but this is not obvious in Fig. 1. The result of a similar analysis of the cell line R3 is shown in Fig. 1b. This cell line was produced from the transfection of full-length mouse vinculin cDNA in an expression vector into γ 229 cells as described in the Methods section. (W. Xu and E.D. Adamson, in preparation). Rescued cells have similar properties to the wild type in that cell-matrix adhesion, cell shape and motility are within the normal range for F9 cells, in contrast to the γ 227 and γ 229 vinculin null cell lines (18). R3 cells show abundant vinculin (Fig. 1b, green) at the cell margins in adhesion plaques and in cell-cell margins that also contain actin (red, and where coincident, yellow). The outlines of cells within the colony where actin and vinculin occur together, are occasionally visible (yellow dashes). The appearance of R3 cells was very similar to wild-type F9 cells (B1M), illustrating the rescue of the phenotype by exogenous vinculin and the correct location of the vinculin in adhesion plaques.

Expression of E-cadherin, α - and β -catenin

In order to insure that the adhesive properties of these cells was due to vinculin and not to clonal differences in adhesive protein levels between the cell lines, we used immunoblotting to

estimate the levels of E-cadherin expressed in B1M, R3, γ 227 and γ 229 cells. Figure 2 shows that the levels were very similar, and cannot account for the difference in cell-cell adhesion demonstrated in this study. The levels of α -catenin and β -catenin, as expected, were also similar. We conclude that differences in the expression of vinculin rather than differences in the expression of cadherins and catenins in the various F9 cells accounts for the differences in the adhesive properties.

Vinculin and Cell-Cell Adhesion

The cell suspension was introduced into a flow channel and cell aggregates were allowed to settle on the fibronectin-coated bottom plate of the flow channel for 15 minutes before the imposition of flow. The flow rate was increased at 20 second intervals so that the wall shear stress acting on the bottom plate of the chamber took the values 0, 7, 21, 35, 49, 63, 77, 91 and 0 dyn/cm². In this method the laminar flow imposed on a cell aggregate that is adherent to the bottom plate of a flow channel exerts peeling forces on the cell-cell adhesion bonds (3). In general, cells in the top layer of an aggregate attached to the bottom plate of a flow channel are exposed to greater levels of shear stress than the lower layers. A cell or a cell aggregate will detach from the parent aggregate when the external fluid force acting on it is large enough to overcome adhesion to neighboring cells.

Cell-cell adhesion strength was measured in aggregates that had been allowed to form from single-cell suspensions for two hours at 37°C. This period is sufficient for most of the cells to

form non-fused aggregates. Fig. 3 shows micrographs depicting the response of a large R3 cell aggregate to the imposed shear flow. Although at higher flow rates the R3 cell aggregate shown in the figure unfolded partially in the direction of flow it remained intact even at the highest level of fluid shear stress used in the experiments (91 dyn/cm^2). In contrast, the $\gamma 227$ cell aggregate shown in Fig. 4 unfolded and aligned in the direction of flow at low levels of flow ($<21 \text{ dyn/cm}^2$). Further increases in flow rate caused extension of cells in the direction of flow failure of cell-cell adhesion bonds and disaggregation. 12 such disaggregation experiments were carried out with each of the B1M, $\gamma 227$, $\gamma 229$ and R3 cell lines and the average number of detachment events per aggregate per cell type, are presented in Fig. 5. Both vinculin null $\gamma 227$ and $\gamma 229$ cell aggregates disaggregated in response to shear flow much more frequently than the vinculin expressing B1M and R3 cell aggregates. These results indicate that at the early to intermediate stages of adhesion (up to 2 hours) the loss of vinculin leads to a very significant reduction in cell-cell adhesion strength.

Next we conducted disaggregation assays on aggregates that were formed in culture for 48 hours. Cell aggregates were collected by vigorously pipetting cells from pre-formed monolayers. Under these circumstances cells will be adherent to one another by mature junctional complexes. These pre-formed aggregates were subjected to the same flow conditions as aggregates that were formed during 2 hours of incubation. The experiments showed that although $\gamma 227$ and $\gamma 229$ cell aggregates detached slightly more frequently from their respective parent aggregates than B1M and R3 cells the differences between the four cell lines in the capacity to resist disaggregation

diminished significantly (Figs. 6,7). These results indicate that with time and the assembly of mature junctional complexes, vinculin-depleted γ 227 and γ 229 cells are able to form cell-cell adhesion bonds comparable in strength to the vinculin-positive B1M and R3 cells.

DISCUSSION

The role of vinculin in motility, substrate adhesion and morphology has been investigated with the use of vinculin-transfected and vinculin-depleted cell lines (4,15,16). Within a few hours of plating vinculin-depletion is associated with increased motility, decreased adhesion to fibronectin-coated surfaces and a more rounded cell morphology (4). However at longer time points, vinculin-null cells formed focal adhesions that were similar to those formed by vinculin-positive cells with the exception that the levels of the focal contact proteins α -actinin, talin and paxillin actually increased in focal adhesions from cells lacking vinculin (20). These results demonstrate that molecular mechanisms for the formation of focal contacts exist, even in the absence of vinculin and indicate that the function of vinculin in cell matrix adhesion may be to increase the rate at which adhesive complexes move between weak and strong adhesive state. Although the mechanism of vinculin action is not well understood these studies clearly point to an important role for vinculin in cell-matrix adhesion. In contrast, even though it is clearly present at cell to cell contact sites no studies have addressed the role of vinculin in intercellular adhesion.

Cadherins are the principle cell surface receptors that mediate cell-cell adhesion in epithelial monolayers (17). Clusters of E-cadherin molecules are concentrated at adherens junctions where they are coupled to the actin cytoskeleton of the cell by the cytoplasmic proteins α and β -catenin and plakoglobin (10). Consistent with this, biophysical studies show that the strength of E-cadherin-mediated cell-cell adhesion is dependent on the efficient coupling of E-cadherin to the

actin cytoskeleton (3). Other studies show that the number of cells which participate in cell-cell adhesive interactions is dependent both on E-cadherin expression and the presence of polymerized actin (1). The role of some adherens junction proteins (e.g. the catenins) in cadherin/actin coupling is quite well established (5). However, no studies have addressed the function of other adherens junction proteins such as vinculin, α -actinin and radixin in cell-cell adhesion. In the present study, we used F9 embryonal mouse carcinoma cells and vinculin-null and vinculin-rescued cell lines derived from them to investigate the role of vinculin in cell-cell adhesion (4).

Since a number of crosslinking molecules coexist at cell-cell contact sites, the role of vinculin in homotypic cell adhesion is likely to be subtle and may be observable only in an adhesion assay where cell aggregates are exposed to large external forces that tend to disaggregate them. In the present study, we adopted a laminar flow assay (disaggregation assay) to examine the role of vinculin on cell-cell adhesion strength. In this method cell aggregates are allowed to attach to the bottom plate of a flow-channel under static incubation and subsequently flow is introduced into the channel on to the aggregates at a specified flow rate and is increased in equal time intervals. The detachment events (cells detaching from the aggregate) are recorded using video microscopy. The numerical simulation of flow past an aggregate shows that fluid shear stress acting on the cell aggregate is highest on the part of the aggregate farthest away from the substrate to which it is adherent, indicating that cells on the top layer are typically subjected to the larger fluid forces (2). When the disaggregation experiments are performed on cell aggregates of comparable size, the average number of disaggregation events is most sensitive to the physical strength of cell-cell

adhesion (3). Therefore, laminar flow assays allow us to measure, for each detachment event, the actual strength of cell-cell adhesion. As described earlier, in laminar flow assays cell-cell separation occurs by a peeling process at the edge of conjugation (3). This approach differs significantly from centrifugation assays in which the force is applied perpendicular to the plane of adhesion and where all adhesion bonds are more or less uniformly stressed (1,14).

In our studies, short-term (2 hours) aggregates of vinculin-null cells disaggregated in response to the imposed shear flow much more frequently than aggregates of wild-type or rescued cells, obtained under identical conditions. Our direct biophysical measurements demonstrated that, at these early stages of aggregate formation, the presence of vinculin increased adhesive strength significantly. Since cell detachment from aggregates occurs by the breaking of cell-cell adhesion bonds these results indicate that the coupling of extracellular adhesion to the cytoskeleton was compromised in vinculin-deficient cells during the early stages of aggregate formation. In contrast, when long-term (48 hours) aggregates were used, systematic differences in cell-cell adhesion strength between vinculin-positive and vinculin-negative cells decreased significantly. This suggests that, with time, the role of vinculin in cell-cell adhesion can be replaced to a certain extent by other membrane-associated proteins and by the assembly of non-vinculin-containing juncyions such as desmosomes. It is possible that the role of vinculin in cell-cell adhesion, as we suggested above for cell-matrix adhesion, is to alter the rate at which adhesive complexes change from low to high adhesive strength.

Although a number of membrane-associated proteins are known to be involved in the linkage of cadherins and integrins to the actin cytoskeleton, the mechanisms involved in this process are not clear. Mechanical principles of adhesion suggest that the ability of F-actin and the cell adhesion molecules that are linked to it (cadherin and integrin receptors) to cluster and form focal points or narrow belts of adhesion is the principal cause of the strengthening of cell-substrate and cell-cell adhesion. In this scheme, strengthening of cell-cell adhesion would occur in three phases. The first phase involves recognition of the cell and spreading driven by the adhesive energy between adjoining cells (1). The second phase involves the initial interaction of adhesion complexes with the actin cytoskeleton. In the third phase a remodeling of the cytoskeleton and aggregation of adhesion complexes in the plane of the membrane acts to anchor the cell onto the substrate through a series of focal points as is the case in cell-matrix adhesion or through a narrow belt as occurs with cell-cell adhesion at the adherens junction. Progression from the first to second phase and from second to third phase coincides with increases in the strength of adhesion. Our model predicts that at the early stages of aggregate formation large numbers of weak adhesive bonds are distributed more or less uniformly along the cell-cell contact plane. However, as in the unzipping of a zipper, it is only the adhesion bonds at the edge of conjugation between the cell and parent aggregate which must resist the resultant fluid force imposed on the cell by laminar flow assays. When this force is large enough to overcome the critical peel tension at the edge of conjugation, these cells peel away from the parent aggregate and gain the speed of flow. Although not mechanically related, another useful analogy is the situation of two glass plates held fast by surface tension. Applying a strong force perpendicular to the plane of adhesion cannot

usually separate the two plates. In contrast, weak shear forces which act to slide the plates along the plane of adhesion can easily separate them.

In the third strengthening phase of adhesion, reorganization of the actin cytoskeleton acts to recruit adhesive complexes to focal points or belts. As described above it is only the adhesion bonds at the edge of conjugation between the cell and parent aggregate which resist the fluid force imposed on the cell. However in this case, the adhesive bonds which were distributed over a wide area at early stages of aggregate formation are now concentrated at the edge of conjugation and are anchored firmly to the cytoskeleton. Consequently, the peel tension required for separation is very high and the shear forces required to detach the cells are enormous. Assuming for the moment that this model is valid how can we explain the the role of vinculin on the development of cell-cell adhesive strength? The effects of vinculin are most evident within two hours of aggregate formation from single cells but vinculin is not required for the formation of aggregates per se. Consequently, it must act either, to increase the coupling strength of individual adhesion complexes between cells during phases one and two, or to increase the rate at which adhesion progresses from phase two to phase three.

At the molecular level an intramolecular association between the 95K head and 30K tail domains of vinculin is thought to mask an F-actin binding site that is present in the tail domain (8). It is possible that at the initial stages of contact formation, vinculin is in its folded state and unable to function as an actin binding and bundling protein. A signal, perhaps a phosphorylation or dephosphorylation event, generated as a result of initial cell-cell adhesion may allow vinculin to

unfold and to interact with actin filaments. This conformation change in vinculin may then act to increase the rate of actin bundling and adhesion complex clustering at cell-cell contact sites (9). In other words, vinculin is not essential for the initial events in cell-cell adhesion, nor is it absolutely required for the formation of adherens junctions, rather it acts, perhaps reversibly, to modulate the rate of transition between the two states.

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FIGURE LEGENDS

Figure 1. Double immunofluorescence staining of actin and vinculin in a) γ 229 vinculin null F9-derived cells, and b) in the same cells that had been transfected with an expression vector for full-length vinculin cDNA (R3 cells see Methods). F-actin is visible (red) because of the specific binding of rhodamine-labeled phalloidin. Vinculin is present in the "rescued" cell line. Clone R3, shown in b) and stained using monoclonal antibodies to human vinculin followed by secondary fluorescein-labeled goat antimouse Ig. The bar denotes 25 mm.

Figure 2. Immunoblot analysis of four components found in cell-cell adhesive plaques. E-cadherin (E-cad), α -catenin (α -cat), β -catenin (β -cat), and β -actin (β -act) was analyzed in lysates of the four cell lines used in these studies. There was no significant difference in the levels of these proteins in the cell lines.

Figure 3. Micrographs showing the physical response of a F9-R3 cell aggregate, formed during two hours of static incubation, to the imposed shear flow. The cell aggregate was allowed to adhere to fibronectin for 15 min under static conditions. Flow was then introduced at 1:40:00 at $t = 7$ dyn/cm² for 20 s and subsequently increased every 20 s to take on the values: 7, 21, 35, 49, 63, and 77 dyn/cm². The cell aggregate remained intact during the course of the experiment. The micrographs A and B show the aggregate before the initiation of flow and in the presence of flow at 77 dyn/cm² respectively.

Figure 4. Micrographs showing the physical response of a γ 227 cell aggregate, formed during two hours of static incubation, to the imposed shear flow. The cell aggregate was allowed to adhere to fibronectin for 15 min under static conditions. Flow was then introduced at 2:32:00 at $t = 7$ dyn/cm² for 20 s and subsequently increased every 20 s to take on the values: 7, 21, 35, 49, 63, and 77 dyn/cm². The cell aggregate disaggregated during the course of the experiment. The micrographs A, B, and C show the shape of the aggregate before the initiation of flow (A), in the presence of flow at 35 dyn/cm² (B), and 35, 49, 63, and 77 dyn/cm² (C), respectively.

Figure 5. Average number of detachment events per cell aggregate per cell type. The figures at the top and bottom, show, respectively, the results for aggregates formed from a single-cell suspension during a two-hour incubation and aggregates formed in culture (pre-formed aggregates). Disaggregation properties of fourteen large cell aggregates (containing at least ten cells) were studied for each case. The bars in the figure indicate standard deviations.

Figure 6. Micrographs showing the physical response of a pre-formed F9-BIM cell aggregates to the imposed shear flow. The cell aggregate were incubated on fibronectin for 15 min under static conditions. Flow was initiated at 3:33:13 at $t = 7$ dyn/cm² for 20 s and subsequently increased every 20 s to take on the values: 7, 21, 35, 49, 63, 77, and 91 dyn/cm². Micrographs A and B show, respectively, the shape of the aggregate before the initiation of flow (A) and in the presence

of flow 91 dyn/cm² (B). Although the cell aggregate elongated extensively in the direction of flow, it remained intact during the course of the experiment.

Figure 7. Micrographs showing the physical response of a pre-formed g229 cell aggregate to the imposed shear flow. The cell aggregate was incubated on fibronectin for 15 min under static conditions (A). Flow was initiated at 5:43:00 at $t = 7$ dyn/cm² for 20 s and subsequently increased every 20 s to take on the values: 7, 21, 35, 49, 63, 77, and 91 dyn/cm². Note that the cell aggregate first aligned itself to the direction of flow and then disaggregated at $t = 35$ dyn/cm² (B).

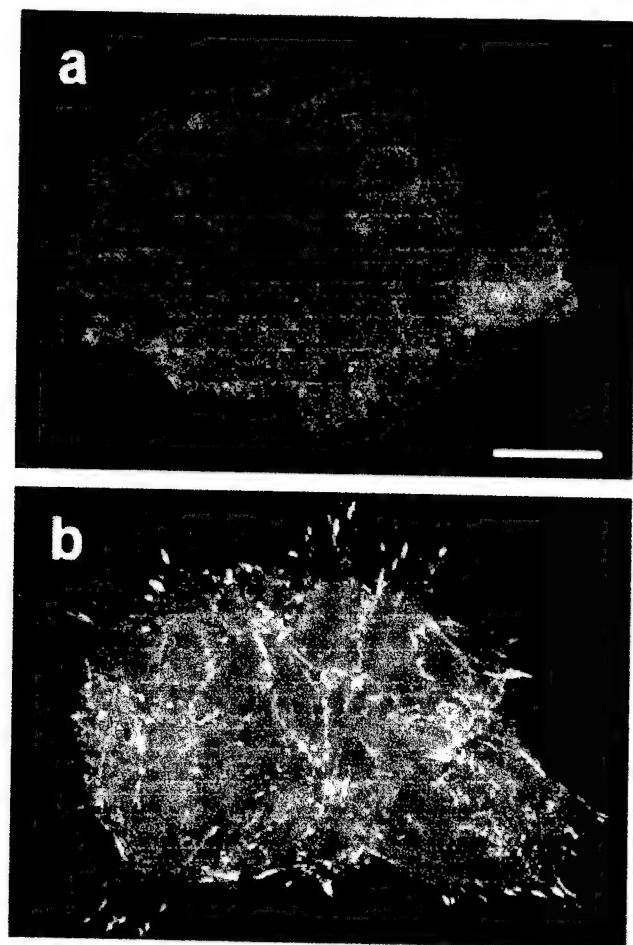


Figure 1

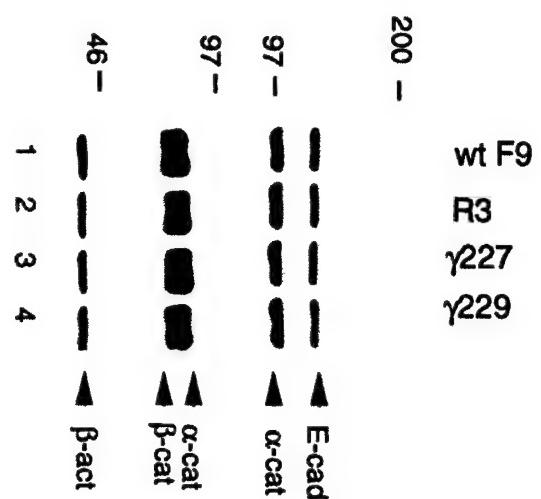


Figure 2

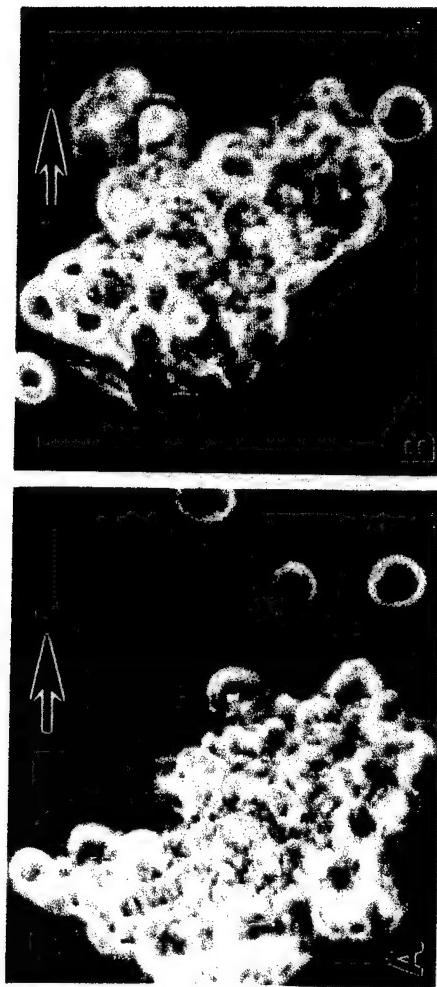


Figure 3

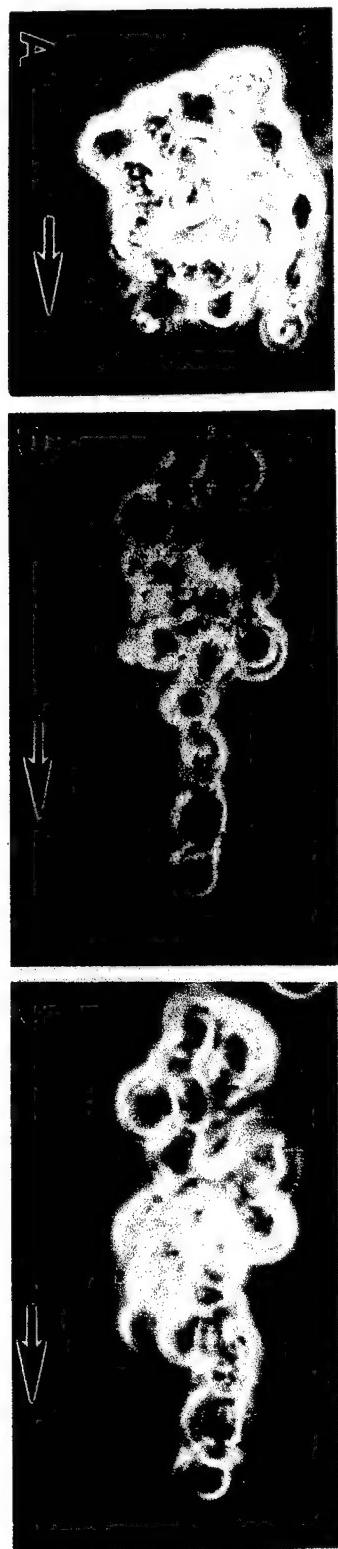


Figure 4

Disaggregation Assay

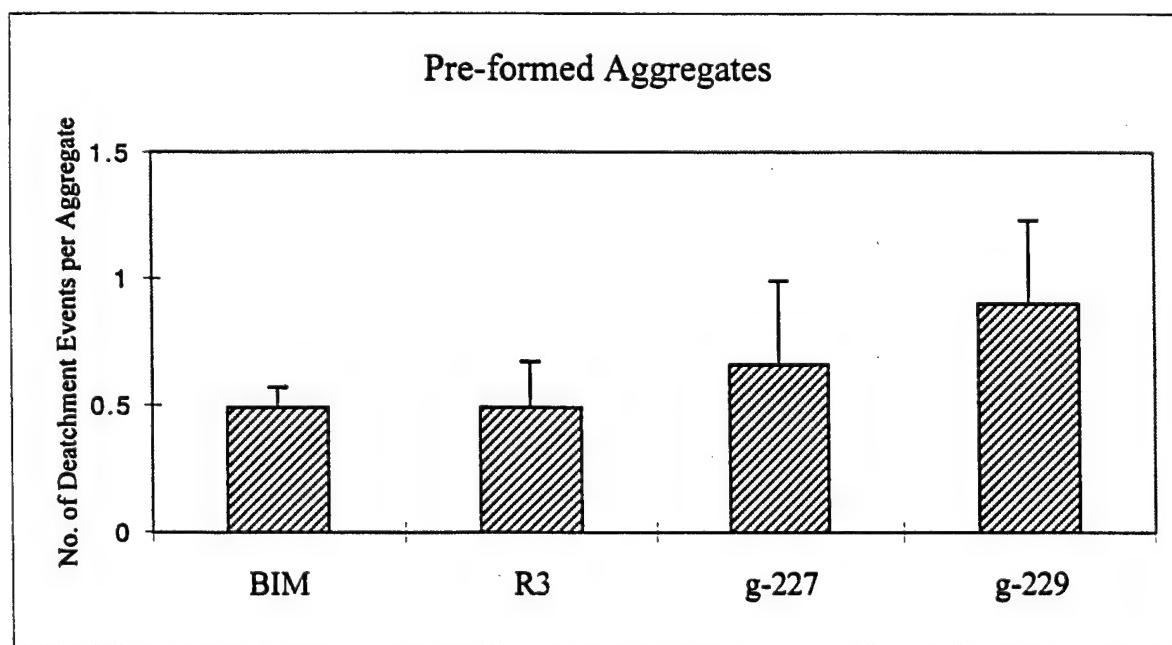
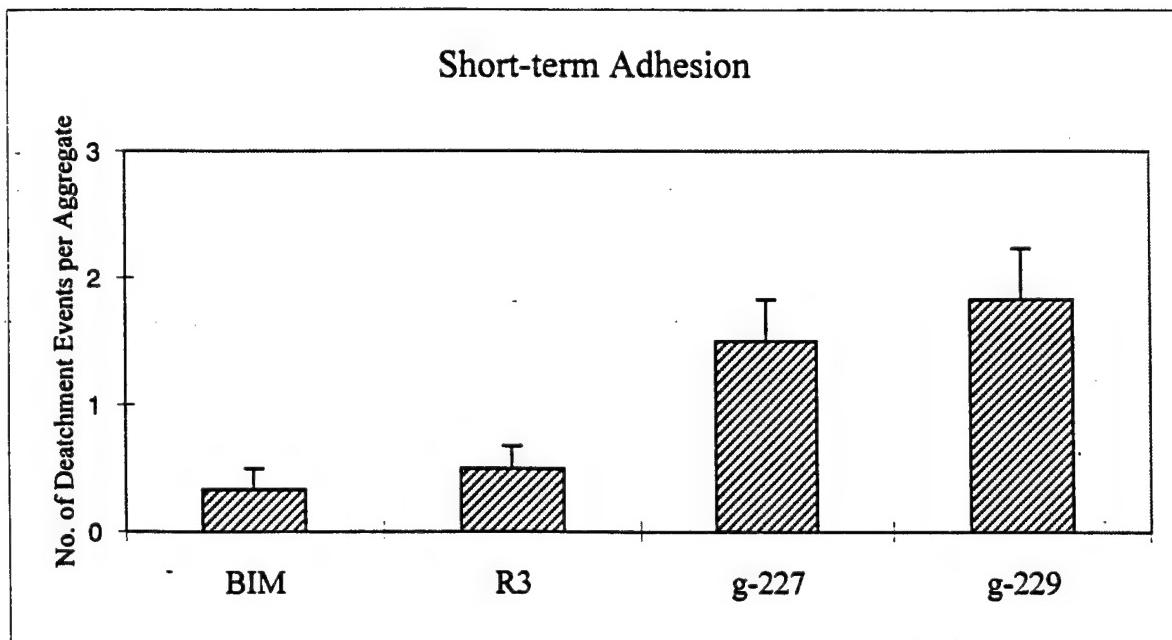


Figure 5

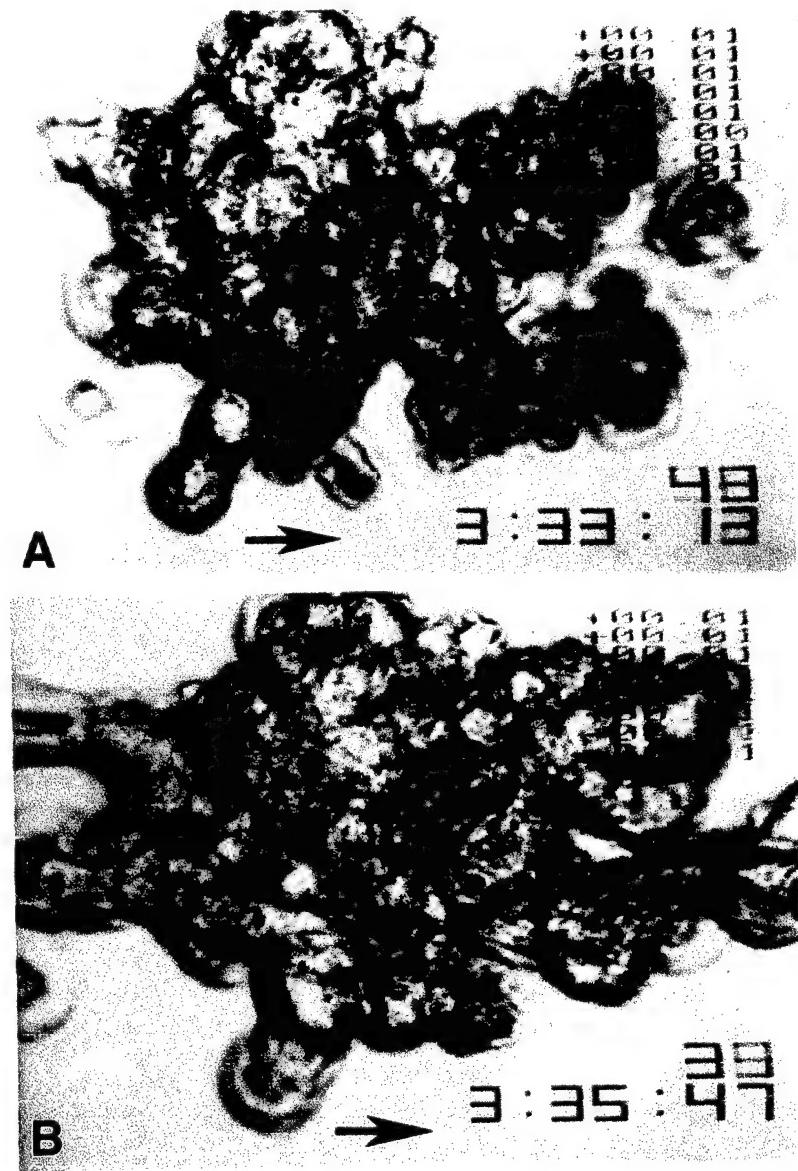


Figure 6

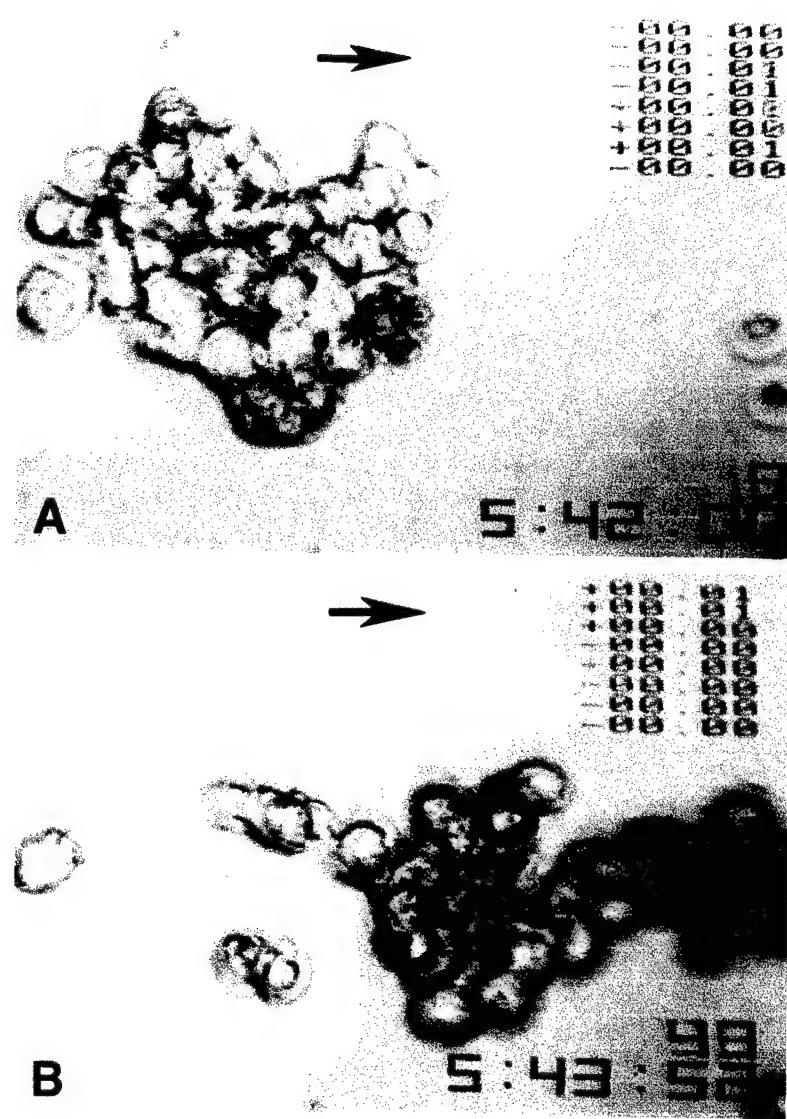


Figure 7

Retinoids Increase Cell-Cell Adhesion Strength, β -Catenin Protein Stability, and Localization to the Cell Membrane in a Breast Cancer Cell Line: A Role for Serine Kinase Activity*

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ABSTRACT

In this study we show that a breast cancer cell line (SKBR3) that expresses no E-cadherin and very low levels of β -catenin protein and exhibits a poorly adhesive phenotype in Matrigel responds to retinoic acid (RA) by a marked increase in epithelial differentiation. Specifically, treatment of cells with all-trans-RA, 9-cis-RA, or a RA receptor α -specific ligand resulted in a large increase in cell-cell adhesive strength and stimulated the formation of fused cell aggregates in Matrigel. A retinoid X receptor-specific ligand was ineffective. Exposure of cells to 9-cis-RA for as little as 4 h was sufficient to maintain the adhesive phenotype for at least 4 days. The effects of 9-cis-RA required protein and RNA synthesis, but were not mediated by factors secreted by stimulated cells or by direct cell contact and did not require serum. These 9-cis-RA-induced morphological effects were completely reversed by growing cells in 50 μ M Ca^{2+} , suggesting a mechanism involving a 9-cis-RA-induced increase in Ca^{2+} -dependent adhesion. Consistent with this, β -catenin protein levels were mark-

edly elevated in the 9-cis-RA-treated cells, and β -catenin became localized to a Triton-insoluble pool at regions of cell-cell contact. No change could be detected in β -catenin steady state messenger RNA levels, but 9-cis-RA did increase β -catenin protein stability. Treatment of cells with low calcium medium did not prevent the 9-cis-RA-induced increase in total β -catenin protein, but did prevent its movement to a Triton-insoluble pool at the cell membrane. Among several kinase inhibitors, only the broad spectrum kinase inhibitor staurosporine and the protein kinase C inhibitor bisindolylmaleimide reversed the morphological changes induced by 9-cis-RA. Like treatment with low calcium medium, these inhibitors did not prevent the 9-cis-RA-induced increase in total β -catenin protein levels, but completely prevented the movement of β -catenin to the cell membrane. These results point to a role for β -catenin and serine kinase activity in mediating the action of 9-cis-RA in epithelial differentiation. (*Endocrinology* 137: 3265–3273, 1996)

CADHERIN-MEDIATED adhesion is fundamentally involved in the organization of epithelial tissues during development, and manipulations of cadherin function result in profound disturbances of tissue organization (1, 2). For cadherins to function in cell-cell adhesion and promote the formation of junctions, several other associated molecules must be expressed (see Ref. 3 for review). These molecules, α -, β -, and γ (plakoglobin)-catenins, link the cadherins to the underlying actin cytoskeleton and are probably involved in propagating adhesion-related signaling. The importance of catenins in mediating adhesion and differentiation is underscored by the findings that the expression of certain catenins is lost in malignant breast carcinoma cells and that the phos-

phorylation state of β -catenin can also influence the transformed phenotype (4, 5).

In previous studies we showed that the expression of certain epithelial markers, including E-cadherin and the catenins, was associated with the differentiation state of a series of breast cancer cell lines (4, 6, 7). Generally, molecular markers of epithelial differentiation were present in normal breast epithelial cells and in well differentiated breast cancer cells, but were reduced or absent in poorly differentiated, highly invasive cells. Highly invasive cells were also inevitably characterized by expression of the mesenchymal intermediate filament protein, vimentin (8). We also described a group of cell lines that were deficient in cell-cell adhesion, but did not express vimentin and were not invasive. These cells were hypothesized to represent an intermediate stage in a spectrum of malignant progression (8). When antibodies to E-cadherin were added to well differentiated lines, they underwent a morphological change to an intermediate phenotype, but did not become invasive. In certain instances exogenous expression of "missing" E-cadherin or catenins into poorly differentiated invasive cancer cells renders the cells

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less invasive, but in other cases it does not (9–11). However, for the most part it is unlikely that the cell dedifferentiation that accompanies malignant progression is the result of a single change in cadherin expression or function. Indeed, this reasoning runs counter to the prevailing dogma that carcinogenesis is a multistage polygenic disease (12, 13). This view of carcinoma as a disease that simultaneously affects several interactive genetic and signaling pathways naturally leads to the conclusion that agents used in the treatment of this group of diseases should also broadly affect multiple (perhaps even alternative or compensatory) pathways. Retinoids are clearly in this category.

Vitamin A and its derivatives (retinoids) are required to support many processes essential to the survival of eukaryotes. These include vision, reproduction, hematopoiesis, bone development, various aspects of metabolism, and pattern formation in development (see Ref. 14 for review). Retinoids also regulate cell proliferation and epithelial differentiation, and are effective in the treatment of several disease states, including certain cancers (14). With the exception of the retinaldehyde/rhodopsin mechanism involved in vision, the retinoids exert their actions at the level of nuclear receptors, which, when activated by retinoids, can modulate the expression of target genes by binding to specific responsive elements in the promoter (15). Once activated, this machinery directly or indirectly regulates the expression of a broad range of genes and signaling pathways. These include growth factors and their receptors, various hormones, cellular enzymes and effectors, matrix proteins and proteolytic enzymes, oncogenes, and transcription factors, such as homeobox genes (14). We now show that retinoic acid (RA) treatment of a breast cancer cell line of the intermediate phenotype results in a dramatic epithelialoid differentiation characterized by a large increase in cell-cell adhesion strength, increased expression of β -catenin protein, and its movement to a Triton-insoluble pool at cell-cell contact sites.

Materials and Methods

Antibodies, retinoids, and other reagents

Polyclonal antibody to β -catenin was a gift from Barry Gumbiner and has been described previously (16). Monoclonal antibody to β -catenin was obtained from Transduction Laboratories (Lexington, KY). 9-cis-RA, the RA receptor- α (RAR α)-specific ligand [4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-anthracenyl)benzoic acid (TTAB), and the retinoid X receptor (RXR)-specific ligand SRI-1217 were gifts from Marcia Dawson (SRI International, Menlo Park, CA) and have been described previously (17, 18). All-trans-RA and the vitamin D₃ analog Ro24-5531 were gifts from Hoffman LaRoche (Nutley, NJ). Ro5531 (1 α ,25-dihydroxy-16-ene-23 yne-26,27-hexafluorocholecalciferol) is 10–100 times more potent than vitamin D₃ in inhibiting breast cancer cell proliferation. Staurosporine and okadaic acid were obtained from Upstate Biotechnology (Lake Placid, NY); cycloheximide, actinomycin D, calpain inhibitor, and heparin were obtained from Sigma Chemical Co. (St. Louis, MO); 5-chloromethyl fluorescein diacetate (CMFDA) was obtained from Molecular Probes (Eugene, OR). Bisindolylmaleimide was obtained from Boehringer Mannheim (Indianapolis, IN). Transforming growth factor- β 1 (TGF β 1) and TGF β latency-associated peptide (LAP) were gifts from R&D Systems (Minneapolis, MN), and Matrigel was a gift from Dr. Hynda Kleinman. Suramin was kindly provided by Dr. Anton Wellstein (Lombardi Cancer Center, Georgetown University, Washington DC), and conditioned medium from MCF-7 cells overexpressing the erbB-2 ligand was a gift from Dr. Ruth Lupu (Lombardi Cancer Center, Georgetown University).

Cells and cell culture

SKBR3 cells, a human breast adenocarcinoma-derived line, were obtained from American Type Culture Collection (Rockville, MD) and maintained in DMEM containing 10% FCS, as described previously (6). For Matrigel morphology experiments, 200 μ l Matrigel were placed into 24-well plates and allowed to gel for 60 min. One hundred thousand cells were plated into each well, and the morphology was monitored after 24–96 h. Generally, the cells were plated in DMEM with 10% FCS containing various concentrations of retinoids or the inhibitors. To address whether the retinoid effects required serum, some experiments were carried out in the absence of FCS. Cell cultures were monitored for up to 4 days. In the coculture experiments, SKBR3 cells were treated for 12 h with 10⁻⁷ M 9-cis-RA, washed, and trypsinized. Another flask of untreated cells was trypsinized at the same time. The 9-cis-RA-treated cells (mostly small aggregates) were labeled with the fluorescent dye CMFDA for 15 min, as described previously, and washed, then equal numbers of treated and untreated cells were plated in 12-well plates containing glass coverslips (4). Once CMFDA entered the cells it was esterified and rendered membrane impermeant; therefore, it could not subsequently pass from one cell to another. Cocultures were monitored for 48 h for any morphological change in the unlabeled cells adjacent to the 9-cis-RA-treated fluorescent cells (see Fig. 2B).

³H]Thymidine uptake

Ten thousand cells were plated into each well of a 24-well plate and allowed to attach overnight. After 48 h of treatment with the agents indicated in Table 1, the cells were labeled with 10 μ Ci/ml [³H]thymidine for 2 h and fixed by adding methanol-acetic acid (3:1) for 1 h. After fixation, the cells were washed in methanol and solubilized by trypsinization (0.5 ml) for 1 h at room temperature; 0.5 ml 1% SDS was added, and the mixture was transferred to scintillation vials. Nine milliliters of Aquamix (ICN, Costa Mesa, CA) were added, and the samples were counted in a liquid scintillation counter.

Laminar flow assays for the measurement of cell-cell adhesion strength

SKBR3 cells that had been exposed to 9-cis-RA for 24 h or control cells were trypsinized in the presence of 5 mM Ca²⁺ resuspended to a density of 10⁶ cells/ml in a volume of 5 ml and allowed to reaggregate for 4 h at 37 °C with shaking at 150 rpm in a T75 flask. Cells were pelleted, and adhesion strength was measured using a laminar flow assay, as described previously (19). Briefly, this involves allowing the cell aggregates to attach to glass coverslips coated with laminin and exposing them to fluid shear stress in a laminar flow chamber. The number of cells that detached from the parent aggregate at a given shear stress was measured, and the adhesion strength was calculated.

Immunoprecipitation, Western blotting, Northern analysis, and immunocytochemistry

Subconfluent cultures in 150-mm dishes were washed twice in isotonic buffer and lysed for 15 min on ice in 1% Nonidet P-40 lysis buffer with 1 mM NaVO₄, 50 mM NaF, 20 μ g/ml phenylmethylsulfonyl fluoride, 2 μ g/ml leupeptin, 1 μ g/ml pepstatin, and 1 μ g/ml aprotinin (20).

TABLE 1. Effects of retinoids, the vitamin D₃ analog Ro5531, and TGF β 1 on [³H]-thymidine uptake by SKBR3 cells

| Treatment | % Inhibition of thymidine uptake after 48 h |
|---|---|
| 9-cis-Retinoic acid (10 ⁻⁷ M) | 95 (0.3) ^a |
| All-trans-retinoic acid (10 ⁻⁷ M) | 98 (0.04) |
| RAR α -specific ligand (10 ⁻⁷ M) | 77 (1.9) |
| RXR-specific ligand (10 ⁻⁷ M) | 16 (0.9) |
| Vitamin D ₃ analog Ro5531 (10 ⁻⁷ M) | 90 (0.3) |
| TGF β 1 (5 ng/ml) | 15 |

Results are expressed as the percent reduction from the nontreated control value. The SD of triplicate values is shown in parentheses.

^a Cell counts after 6 days were reduced by 75%.

After clarification for 15 min at top speed in a microfuge at 4°C, lysates were precleared with 100 μ l protein A-Sepharose (Pharmacia, Piscataway, NJ) and 20 μ l normal rabbit serum twice for 1 h each time. Protein content was measured by a colorimetric method (Bio-Rad Laboratories, Richmond, CA). Equal amounts of protein (~1 mg) were incubated with 2 μ l anti- β -catenin rabbit antiserum or nonimmune serum. Immune complexes were precipitated with 10 μ l protein A-Sepharose, washed six times in lysis buffer, and resuspended in loading buffer. Western blotting was carried out as described previously, using either the polyclonal β -catenin antibody at a 1:5000 dilution or the monoclonal antibody (Transduction Laboratories). On some occasions cells were extracted directly in reducing sample buffer as described previously, except that the samples were boiled for 30 min before SDS-PAGE (4). The extended boiling (increased from 5 to 30 min) in the presence of reducing agent significantly reduced the viscosity of the samples, making treatment with deoxyribonuclease or sonication unnecessary.

Immunocytochemistry was carried out with or without Triton extraction as described previously (4). Total RNA was isolated from SKBR3 cells at various times after treatment with RA as described previously (6). Blots were probed with a random primer-labeled EcoRI fragment from the coding region of human β -catenin.

Results

RA promotes SKBR3 cell epithelial differentiation and increases cell-cell adhesion strength

Morphology on plastic. We showed previously that untreated SKBR3 cells growing on plastic express no E-cadherin and very low levels of β -catenin protein, and exhibit a rounded morphology (4, 6). After treatment with 9-cis-RA, all-trans-RA (ATRA) exhibited a marked change in appearance, characterized by an increase in cell-cell and cell-substratum contact (Fig. 1). After 2–4 days of 9-cis-RA or ATRA treatment, SKBR3 cells were also growth inhibited (Table 1). TTAB, a retinoid analog known to specifically activate RAR α , also promoted the morphological change and growth inhibition, whereas SRI-1217, a retinoid derivative that only activates RXRs, did not (see Refs. 17 and 18 for a more detailed explanation of ligand specificities). In all of the experiments discussed here, 9-cis-RA and ATRA gave similar results; therefore, only the results with 9-cis are presented unless otherwise indicated. ATRA is known to activate RAR in heterodimeric complexes with RXRs. 9-cis-RA also activates RAR/RXR heterodimers, as it is readily converted to ATRA, but also activates RXR homodimers. SKBR3 cells were also growth inhibited by the vitamin D₃ analog Ro24-5531 (Table 1). However, this agent did not result in the pronounced morphological change observed after RA treatment (Table 2). SKBR3 cells are known to overexpress the tyrosine kinase receptor erbB-2 and to respond to high levels of the erbB-2 ligand with decreased proliferation and an increase in the expression of breast-specific differentiation markers (21–24). Treatment of SKBR3 cells with conditioned medium containing erbB-2 ligand resulted in a morphological change, as described previously (21). Cells were flatter, indicating an increase in cell-substratum contact, and had many extended processes. However, there was no evidence of increased cell-cell contact (Table 2).

Matrigel morphology and laminar flow assays. In previous studies we showed that SKBR3 cells exhibited a rounded appearance when growing in the basement membrane gel Matrigel and did not form fused cell colonies as was the case with other, more differentiated lines such as MCF-7. After 12 h of 9-cis-RA treatment, SKBR3 cells formed fused cell

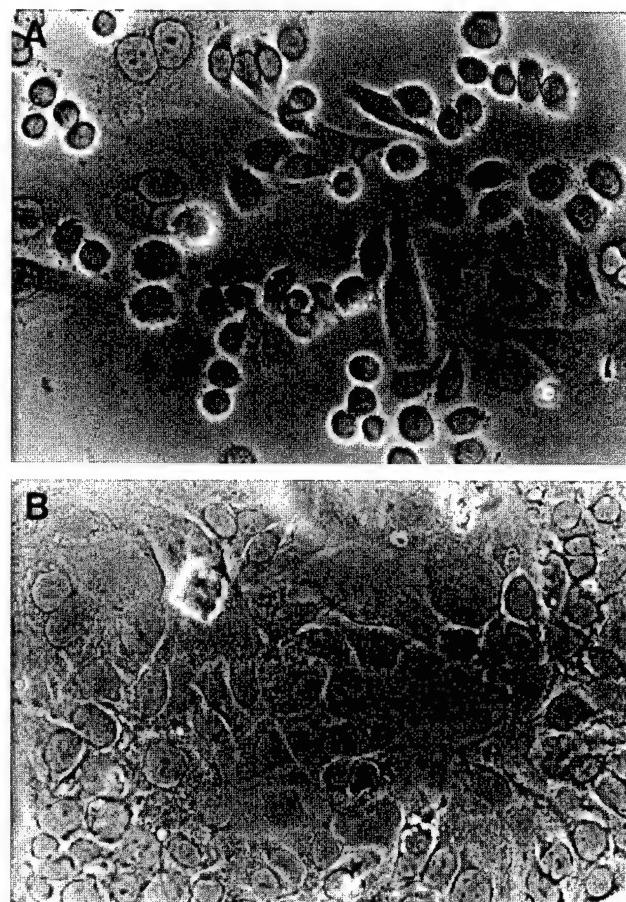


FIG. 1. 9-cis-RA promotes epithelial differentiation. Breast cancer cells (SKBR3) were treated for 24 h with 10^{-7} M 9-cis-RA. Control SKBR3 cells exhibit a rounded morphology (A). After treatment with 9-cis-RA, cells exhibited a marked change to a flattened epithelial morphology (B).

colonies indistinguishable from those formed by MCF-7 or T47D cells (Table 2 and Fig. 2A). No effect on Matrigel morphology was observed if the cells were treated with Ro5531, TGF β , or erbB-2 ligand, indicating a specificity for 9-cis- or ATRA (Table 2). The fused morphology in Matrigel is generally assumed to represent an increase in cell-cell adhesion strength, although no studies have actually addressed this. We recently developed methodology with which to investigate the strength of cell-cell adhesion directly (19). Using this laminar flow assay we found that 9-cis-RA treatment of SKBR3 cells resulted in a dramatic increase in the strength of cell-cell adhesion from less than 7 to 100 dyn/cm².

Effects of RA on SKBR3 cell epithelial differentiation require RNA and protein synthesis, but not serum

Not surprisingly, treatment of SKBR3 cells with actinomycin D or cycloheximide prevented the effects of 9-cis-RA on Matrigel morphology, indicating a requirement for RNA and protein synthesis (Table 2). Removal of serum from the medium followed by five washes in serum-free medium did not affect Matrigel morphology after 9-cis-RA treatment, indicating that factors present in serum are not necessary for 9-cis-RA to exert its effect on the SKBR3 phenotype (Table 2).

TABLE 2. Summary of the effect of retinoids and other agents on SKBR3 morphology in Matrigel, β -catenin protein levels and localization in a Triton insoluble pool at the cell membrane

| Treatment | Matrigel morphology | β -cat protein levels | Triton-insoluble β -cat |
|--|---------------------|-----------------------------|-------------------------------|
| None | Separated | Low | Low |
| 9cRA, ATRA (10^{-8} – 10^{-6} M) | Fused | High | High |
| RAR α ligand (10^{-8} – 10^{-6} M) | Fused | High | High |
| RXR ligand (10^{-8} – 10^{-5} M) | Separated | Low | Low |
| 9cRA + actinomycin D | Separated | ND | ND |
| 9cRA + cycloheximide | Separated | ND | ND |
| 9cRA + staurosporine (10 nM) | Separated | High | Low |
| 9cRA + bisindoylmal. (10 μ M) | Separated | High | Low |
| 9cRA + low calcium (50 μ M) | Separated | High | Low |
| 9cRA + okadaic acid (20 nM) | Fused | High | High |
| 9cRA + no serum | Fused | High | High |
| 9cRA + heparin (20–200 μ g/ml) | Fused | ND | ND |
| 9cRA + suramin (50 μ M) | Fused | ND | ND |
| 9cRA + genistein | Fused | ND | ND |
| 9cRA + herbimycin | Fused | ND | ND |
| Conditioned medium ^a | Separated | ND | ND |
| Cell-cell contact ^b | Separated | Low | Low |
| erbB-2 ligand | Separated | Low | Low |
| TGF β (5 ng/ml) | Separated | ND | ND |
| TGF β LAP (125 μ g/ml) | Separated | ND | ND |
| Okadaic acid (20 nM) | Separated | Low | Low |

TGF β LAP, TGF β latency-associated peptide; ND, not determined.

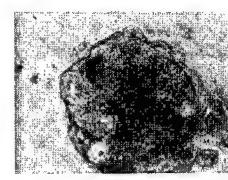
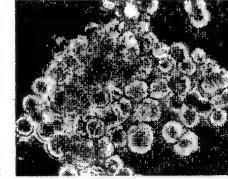
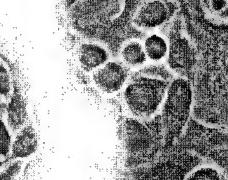
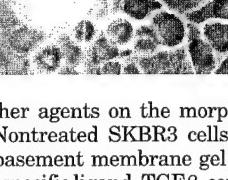
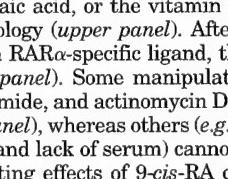
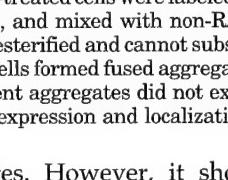
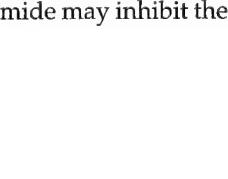
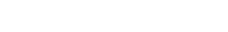
^a Cells were treated with RA for 24 h, then washed five times, and medium collected during the following 24 h was added to untreated cells.

^b RA-treated cells were labeled with the dye CMFDA, trypsinized, and mixed with non-RA-treated cells. CMFDA cannot pass between cells. Only fluorescent cells formed fused aggregates. Nonfluorescent cells adjacent to fluorescent aggregates did not exhibit any changes in morphology or β -catenin expression and localization.

Effects of RA on SKBR3 cell epithelial differentiation are blocked by low calcium medium and the serine kinase inhibitors staurosporine and bisindoylmaleimide

The effects of 9-cis-RA on Matrigel morphology could be reversed by growing the cells in medium containing 50 μ M Ca²⁺, indicating a mechanism involving Ca²⁺-dependent adhesion (Table 2 and Fig. 2A). The broad spectrum kinase inhibitor staurosporine (10 nM for 16 h) also reversed the effects of 9-cis-RA on Matrigel morphology, whereas the serine phosphatase inhibitor okadaic acid was without effect when used alone (Table 2). Staurosporine is a broad spectrum kinase inhibitor that can inhibit serine kinases such as protein kinase C (PKC), cAMP, and cGMP-dependent kinases, as well as certain receptor and nonreceptor tyrosine kinases, such as *src* and epidermal growth factor receptor, at the doses used in this study. To further define the role of kinase activity in mediating the effects of 9-cis-RA, we tested the effects of the tyrosine kinase inhibitors herbimycin and genistein and the specific PKC inhibitor bisindoylmaleimide (25). Neither herbimycin nor genistein affected the Matrigel morphology of SKBR3 cells (Table 2). Bisindoylmaleimide (10 μ M) completely reversed the 9-cis-RA-induced morphological changes (Table 2 and Fig. 2A). This agent is quite specific for PKC; the inhibition constants for other known serine/threonine kinases and tyrosine kinases are at concentrations 2–6 orders of magnitude higher than those required to block PKC (25). These results point to a role for serine kinase activity, possibly PKC, in mediating some aspect of the 9-cis-RA-

A Treatment Matrigel Morphology

| | |
|---|---|
| Control |  |
| RXR-specific ligand |  |
| TGFβ |  |
| Cond. medium |  |
| Okadaic acid |  |
| Vitamin D |  |
| | |
| 9-cis RA, ATRA |  |
| RARα-specific ligand |  |
| RA+tyr. kinase inhibitors |  |
| RA+suramin/heparin |  |
| RA-serum | |

B

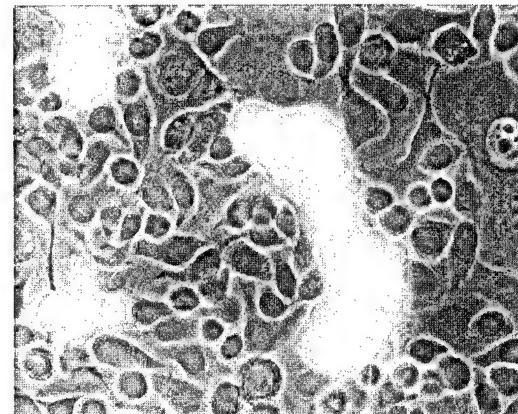


FIG. 2. A, The effects of RA and other agents on the morphology of SKBR3 cells growing in Matrigel. Nontreated SKBR3 cells grow as aggregates of nonfused cells on the basement membrane gel Matrigel (upper panel). Treatment with a RXR-specific ligand, TGF β , conditioned medium from RA-treated cells, okadaic acid, or the vitamin D analog R5531 did not influence this morphology (upper panel). After 24 h of treatment with 9-cis-RA, ATRA, or a RAR α -specific ligand, the aggregates consist of fused cells (middle panel). Some manipulations (e.g. staurosporine, low calcium, cycloheximide, and actinomycin D) can prevent this phenotypic change (lower panel), whereas others (e.g. heparin, suramin, tyrosine kinase inhibitors, and lack of serum) cannot (middle panel). B, The differentiation-promoting effects of 9-cis-RA cannot be transmitted directly to other cells. RA-treated cells were labeled with the fluorescent dye CMFDA, trypsinized, and mixed with non-RA-treated cells. Once inside the cell, CMFDA is esterified and cannot subsequently pass between cells. Only fluorescent cells formed fused aggregates. Nonfluorescent cells adjacent to fluorescent aggregates did not exhibit any changes in morphology or β -catenin expression and localization.

induced morphological changes. However, it should be pointed out that bisindoylmaleimide may inhibit the activity of an unknown kinase.

Differentiation-promoting effects of 9-cis-RA do not appear to be transmitted directly to other cells

In other systems, retinoids exert some of their effects through the increased expression of secreted proteins, such as TGF β (26–28). Although TGF β alone is not responsible for the epithelial differentiation observed in our experiments (Table 2), it is still possible that TGF β is required (but not sufficient) for the epithelial differentiating effects of RA. To test this, cells were treated with 9-cis-RA in the presence of LAP at a concentration 5× greater than that required to completely block the action of TGF β in other TGF β -responsive breast cancer cell lines (our unpublished results). Table 2 indicates that LAP does not reverse the effects of 9-cis-RA on Matrigel morphology. This experiment excludes a role for TGF β in mediating the effects of 9-cis-RA, but does not rule out some other secreted factor. To test this, cells were treated with 9-cis-RA (10^{-7} M) for 4 h, the minimum time required for morphological changes to be observed (after 16 h). Cells were washed (five times), and a small volume (5 ml for a T75 flask) of fresh medium without 9-cis-RA was added. Twenty-four hours later, conditioned medium was collected, centrifuged to remove cell debris, and added to fresh cells. The cells that had been treated with 9-cis-RA for only 4 h underwent the characteristic morphological change after 16 h. However, cells that were treated with conditioned medium collected from these cells did not exhibit epithelial differentiation (Table 2). As it is possible that the effects of 9-cis-RA may be mediated by a cell surface molecule or one with limited local diffusion, perhaps something acting via the extracellular matrix, we performed coculture experiments similar to those carried out by Brown *et al.* in their studies of the local paracrine effect of the *wingless* gene product (29). To conclusively differentiate 9-cis-RA-treated cells from untreated cells, we took advantage of the dye CMFDA, which we used previously in a similar situation (4). Once it has entered cells, CMFDA is esterified and cannot pass through the cell membrane or gap junctions to other cells, thus acting as a permanent cell marker. We labeled 9-cis-RA-treated cells with CMFDA and cocultured them with untreated cells as described in *Materials and Methods*. After 24 h of coculture, the 9-cis-RA-treated cells were clearly visible as fluorescent cell aggregates in a field of nonfluorescent cells (Fig. 2B). If the effects of 9-cis-RA were transmitted via the cell surface or via a molecule with limited diffusion potential, we would have expected a marked morphological change in non-9-cis-RA-treated cells adjacent to the fluorescent aggregates. This was not the case. Although the fluorescent cells previously treated with 9-cis-RA maintained their characteristic morphology for up to 4 days, there was no obvious change in the appearance of adjacent or more distant cells that had not been treated with 9-cis-RA (Table 2 and Fig. 2B).

9-cis-RA increases β -catenin protein levels and movement to a Triton-insoluble pool at cell-cell contact sites, but does not increase steady state messenger RNA (mRNA) levels

We showed previously that SKBR3 cells do not express E-cadherin, but that increased staining of an E-cadherin-like molecule at the adherens junction occurred upon treatment with 9-cis-RA (6, 30). Pierceall *et al.* (31) demonstrated that

SKBR3 cells have a homozygous deletion of the E-cadherin gene. This accounts for the lack of E-cadherin immunoreactivity observed in our earlier studies in which we used a monoclonal antibody and indicates that the molecule that was influenced by 9-cis-RA and detected by a polyclonal antibody is not E-cadherin (30). Others have reported that SKBR3 cells express detectable amounts of E-cadherin RNA, as assessed by Northern analysis (32). Although the identity of the E-cadherin-like SKBR3 cadherin remains a mystery, the demonstration that it becomes membrane associated after 9-cis-RA treatment suggests a change in the expression or function of a cadherin-associated molecule necessary for its localization at the cell junction. We showed previously that SKBR3 cells express normal levels of the cadherin-associated proteins α -catenin and plakoglobin, but expressed very low levels of β -catenin protein. In the present study we also show that SKBR3 cells express low levels of β -catenin protein (Figs. 3 and 4). Immunostaining confirmed that most SKBR3 cells were completely negative for β -catenin; however, approximately 5% of the cells were strongly positive (Fig. 3). In the few positive cells, β -catenin was diffusely distributed throughout the cytoplasm and was not restricted to the cell membrane or cell-cell contact sites. After 9-cis-RA treatment, there was a marked increase in the overall staining intensity of β -catenin. Most of the β -catenin was present at the cell membrane at points of cell-cell contact. Although it was present at the adherens junction, it was not restricted to that location; instead, β -catenin was present throughout the basolateral membrane of the cells. The membrane-associated β -catenin was stable to Triton extraction, indicating that it was linked to the actin cytoskeleton (Fig. 3C). As we have not identified the SKBR3 cadherin, we do not have reagents with which to investigate directly cadherin/catenin interactions in SKBR3 cells and cannot determine whether 9-cis-RA alters the association of β -catenin with the SKBR3 cadherin. The increased β -catenin protein expression observed by immunocytochemistry was confirmed by Western analysis (Fig. 4). Maximum β -catenin protein levels (10 times the control value) were observed after 48 h of treatment with RA at a concentration of 10^{-6} M. In contrast, RA had no effect on β -catenin steady state mRNA levels (Fig. 4C).

9-cis-RA increases β -catenin protein stability

The increased β -catenin protein levels that occur after RA treatment could be the result of increased protein synthesis or decreased degradation. To test this, we took advantage of the ability of calpain inhibitor to reversibly prevent β -catenin protein degradation in SKBR3 cells (our unpublished observations). SKBR3 cells treated with calpain inhibitor for 4 h accumulated β -catenin protein (Fig. 5, A and B). The level of β -catenin present after this treatment was the same in control cells and cells treated for 48 h with 9-cis-RA, indicating that 9-cis-RA does not increase the rate of β -catenin protein synthesis. After a chase in medium without calpain inhibitor, the amount of β -catenin present in control cells decreased rapidly, with a half-life of less than 1 h, and no protein was detectable after 8 h (Fig. 5A). In contrast, in cells treated with 9-cis-RA, the β -catenin half-life was approximately 2 h, with protein still detectable after 24 h of chase (Fig. 5B). These

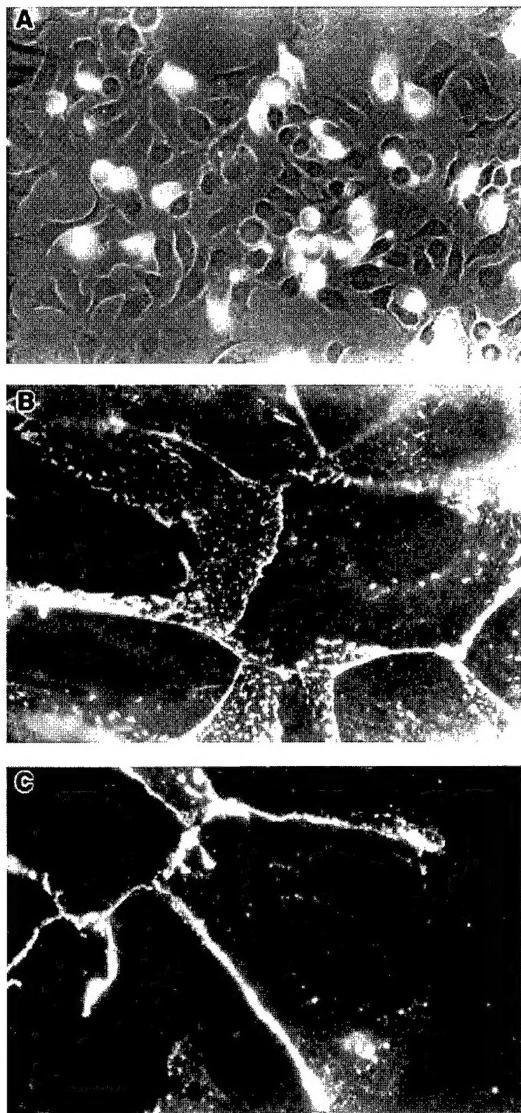


FIG. 3. 9-cis-RA increases cellular β -catenin staining and movement to a Triton-insoluble pool at cell-cell contact sites. **A**, Control SKBR3 cells express very low levels of β -catenin. Most cells are completely negative, but approximately 5% of the cells are brightly positive. In this selected field that contains more positive cells than most fields, the β -catenin in the positive cells is diffusely distributed and not present at cell-cell contact sites. **B**, Treatment with RA results in a dramatic increase in β -catenin staining of all cells. β -Catenin in 9-cis-RA-treated cells is largely restricted to areas of cell-cell contact (**B**) and is insoluble in Triton (**C**).

results are similar to those observed for the *wingless*-mediated stabilization of armadillo protein in *Drosophila* and prompted us to investigate whether the effects of 9-cis-RA in our system are mediated by a member of the *wnt* family (33).

*Effects of 9-cis-RA are not mediated by a member of the *wnt* family*

wnt-1/wingless effects are exerted locally, perhaps via the extracellular matrix, and are the result of increased armadillo protein stability (33). Although the experiments described above do not point to a role for such local interactions in mediating the effects of 9-cis-RA on Matrigel morphology

(Table 2), we further addressed the potential role of *wnt-1* in mediating the effects of 9-cis-RA on SKBR3 Matrigel morphology and β -catenin protein levels by testing the effects of a known inhibitor of *wnt-1* action, heparin (29). Table 2 shows that heparin did not affect the ability of 9-cis-RA to mediate a morphological change. Although heparin is a potent inhibitor of the action of *wnt-1*, it is less effective at inhibiting other *wnt* family members. However, suramin, which is an effective inhibitor of other *wnts* and many other growth factors, did not influence the effects of 9-cis-RA. These results strongly suggest that the effects of 9-cis-RA to increase β -catenin protein levels and stimulate SKBR3 epithelial differentiation are not mediated by a member of the *wnt* family.

Low calcium medium and serine kinase inhibitors do not affect 9-cis-RA-increased β -catenin protein levels, but do prevent β -catenin movement to the cell membrane

As staurosporine, bisindolylmaleimide, and low calcium medium were able to reverse the effects of 9-cis-RA on epithelial differentiation, we expected that the 9-cis-RA-induced increase in β -catenin protein levels would be inhibited by these treatments. However, treatment with the kinase inhibitors or with low calcium medium did not prevent the 9-cis-RA-mediated increase in β -catenin levels, as demonstrated by immunoblotting (Figs. 6 and 7). Immunocytochemistry revealed high cytoplasmic levels of β -catenin under these conditions, but little β -catenin was observed at the cell-cell contact sites (Fig. 6). These results indicate that serine kinase activity and/or the function of a calcium-dependent adhesion molecule are necessary to localize β -catenin efficiently at the cell membrane. As stated above, at this time we cannot investigate directly whether serine kinase inhibitors influence cadherin/ β -catenin association in SKBR3 cells.

Discussion

In this study we demonstrate a relationship between retinoid-induced differentiation and the functional expression of components of the cadherin/catenin-based adhesion and signaling system in breast cancer cells. β -Catenin is certainly required for cadherin-based cell-cell adhesion; however, the demonstration that β -catenin associates with the tumor suppressor gene *APC* in the absence of E-cadherin points to an additional role for β -catenin in the control of cell proliferation (34–36). β -Catenin is also a substrate for at least one serine kinase as well as for several receptor and nonreceptor tyrosine kinases (5, 36) (Lechleider, R., K. Orford, C. L. Sommers, and S. W. Byers, submitted). Collectively, these data strongly indicate that retinoids, cadherins and catenins, and serine and tyrosine kinases are intricately involved in the homeostasis of the epithelial phenotype. Consequently, it is likely that direct or indirect perturbations of any of these systems could lead to alterations in the control of cell proliferation and the epithelial phenotype, resulting in the development or progression of cancer.

The finding that a ligand that specifically activates RAR α (TTAB) is able to duplicate the effects of all-trans-RA and 9-cis-RA in stimulating the adhesive phenotype and inhibiting thymidine incorporation and cell proliferation *in vitro* unequivocally demonstrates the involvement of this class of

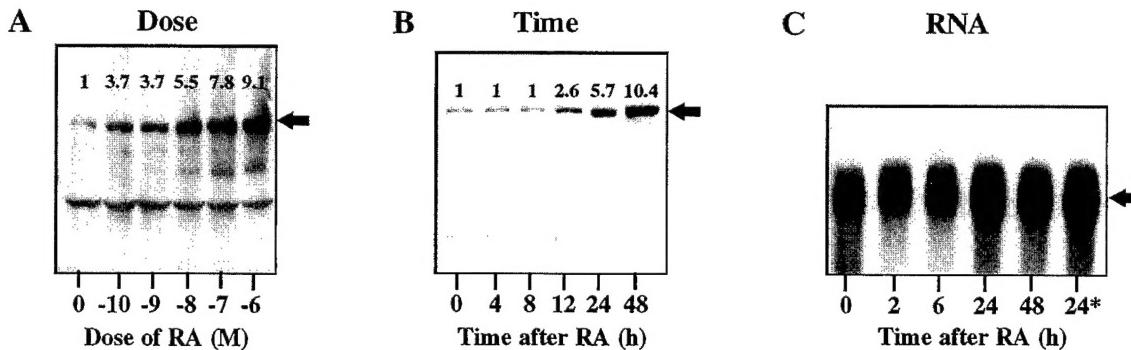


FIG. 4. 9-cis-RA increases β -catenin protein levels in a dose- and time-dependent manner, but has no effect on β -catenin steady state RNA. A, Cellular β -catenin levels were measured by Western blot analysis of SKBR3 cell detergent lysates after treatment with 10^{-10} - 10^{-6} M RA for 24 h. Maximal stimulation was observed after treatment with 10^{-6} M RA. Numbers above the indicated β -catenin band indicate the fold increase from control, as measured by densitometry. B, Time course of the effects of 10^{-7} M 9-cis-RA on β -catenin protein levels. Maximum stimulation was observed 48 h after treatment, and levels remained elevated for at least 4 days. C, Time course of the effects of 10^{-7} M 9-cis-RA on β -catenin mRNA levels. No significant changes in β -catenin mRNA were observed after treatment with RA. *, Cells were treated for 24 h with the RXR-specific ligand.



FIG. 5. RA increases β -catenin protein stability. Control cells (A) or cells treated with RA for 24 h (B) were exposed to calpain inhibitor ($10 \mu\text{M}$) for 4 h, then chased in medium without calpain inhibitor for the indicated times. Nonidet P-40 cell lysates were probed by Western blot for β -catenin. The arrowhead indicates the β -catenin band; the large lower mol wt band present in all lanes is a nonspecific band derived from the serum present in the chase medium.

RAR. As RAR homodimers are not thought to be biologically relevant, we ascribe this activity to heterodimeric RAR α complexes with a member of the RXR class of receptors (15). Unlike RAR, RXR homodimers can mediate ligand-dependent actions. However, a ligand that specifically activates RXR/RXR homodimers does not mediate retinoid-induced epithelial differentiation in this system.

What is the mechanism of 9-cis-RA induction of SKBR3 cell epithelial differentiation?

The effects of retinoids could be observed if the cells were treated for as little as 4 h, which infers a switch-like mechanism of action. That is, in this case, 9-cis-RA may turn on a (genetic) program that, once initiated, maintains the epithelial phenotype. Inhibition of RNA synthesis by actinomycin prevented these effects, indicating a role for transcriptional activation. A recent study also shows an effect of ATRA on the adhesive phenotype of a cancer cell line (37). However, the mechanism of action of ATRA is quite different from that observed here. In that study, ATRA induced the adhesive function of endogenous E-cadherin in an adhesion-defective cell line within 30 min. No changes in the levels of β -catenin protein were observed, and the effects of ATRA did not require RNA or protein synthesis. In contrast, the results of the present study are reminiscent of the effects of retinoids

in development. In developing systems and other situations, retinoids act in part through the induction of expression of secreted molecules such as TGF β , the TGF β family member decapentaplegic, *wingless*, and hedgehog (27, 28, 38, 39). The possibility that the effects of 9-cis-RA in our experiments are mediated via an indirect action of a secreted *wingless*-like molecule are particularly interesting, as exogenous expression of *wingless* or its mammalian homolog *wnt-1* results in increased β -catenin and plakoglobin protein expression and increased cell-cell adhesion (40, 41). Breast cancer cells are also known to express several members of the *wnt* family (42). However, conditioned medium (concentrated up to 10 times) from 9-cis-RA-treated cells was ineffective in mediating epithelial differentiation. Treatment of SKBR3 cells with TGF β 1 did not result in changes in β -catenin expression or epithelial differentiation. This probably rules out an effect of 9-cis-RA-induced secretion of certain molecules, such as TGF β , but does not exclude a role for *wnt*-related proteins, which have very limited diffusion and are thought to exert their action locally. However, heparin and suramin, which are known to reverse the effects of *wingless* and other *wnt* family members, did not reverse the 9-cis-RA-induced changes. To generally investigate the possibility that the 9-cis-RA-induced effects can be transmitted to neighboring cells, we cocultured 9-cis-RA-treated cells with untreated cells and looked for an effect on cells within a 1–10 cell radius. These experiments are similar in design to those used by Jue and co-workers (29) to demonstrate that *wnt-1*-transfected cells could transform neighboring nontransfected cells. In our experiments we were unable to morphologically transform neighboring non-9-cis-RA-treated cells by coculturing fluorescently tagged 9-cis-RA-treated cells with untreated cells. Although we cannot exclude the possibility that 9-cis-RA-treated cells stop producing any putative mediators of epithelial differentiation after 4 h (the time of incubation with 9-cis-RA), taken together these results raise the possibility that the effects of 9-cis-RA on epithelial differentiation may be autonomous and not transmitted by secreted or cell surface molecules.

The effects of 9-cis-RA on β -catenin expression are not



FIG. 6. Staurosporine and low calcium medium prevent the movement of RA-induced β -catenin to cell-cell contact sites. **A**, β -Catenin levels in control cell are very low. After treatment with 10^{-7} M 9-cis-RA plus staurosporine (**B**) or with 10^{-7} M 9-cis-RA in low calcium medium (**C**), β -catenin staining is still elevated, but β -catenin is not present at cell-cell contact sites. The *inset* in **C** represents a Western analysis of β -catenin levels in detergent lysates of cells growing in normal and low calcium-containing media. Lane 1, 220-, 97-, and 66-kDa standards; lane 2, control without 9-cis-RA; lane 3, with 9-cis-RA; lane 4, low calcium control; lane 5, low calcium with 9-cis-RA.

mediated by an increase in the levels of β -catenin steady state RNA, ruling out a role for direct regulation of the β -catenin gene by 9-cis-RA. The increased protein levels observed after 9-cis-RA treatment are the result of decreased degradation of β -catenin protein. As the effects of RA can be reversed by actinomycin D, these results indicate that 9-cis-RA might positively or negatively regulate the expression of genes that directly or indirectly affect β -catenin protein degradation. There are several possible mechanisms by which 9-cis-RA

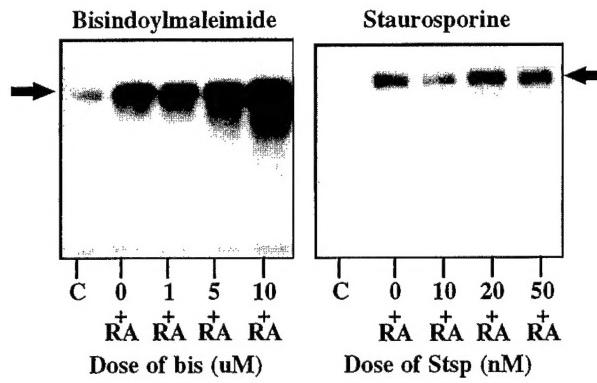


FIG. 7. The PKC inhibitor bisindolylmaleimide and the broad spectrum kinase inhibitor staurosporine do not reverse the effects of 9-cis-RA on β -catenin protein levels. *Left panel*, Bisindolylmaleimide, a specific PKC inhibitor, does not prevent the 9-cis-RA-induced increase in β -catenin protein levels. At $10 \mu\text{M}$, bisindolylmaleimide augments the effects of 10^{-7} M RA. *Right panel*, Staurosporine does not prevent the effect of 9-cis-RA on β -catenin protein levels. Western blot analysis was used.

could increase β -catenin stability. For example, 9-cis-RA might increase the expression of a β -catenin binding protein in the membrane (a cadherin?), which could sequester newly synthesized β -catenin protein into a pool with a longer half-life. Alternatively, 9-cis-RA could increase the expression of genes that directly modulate β -catenin stability in the cytoplasm. It is also interesting to note that the β -catenin-associated protein APC is able to direct β -catenin protein for degradation (43). Whether 9-cis-RA influences the expression or function of a cadherin or APC remains to be determined.

Treatment of cells with the kinase inhibitors staurosporine and bisindolylmaleimide reversed the effects of 9-cis-RA on epithelial differentiation and cell-cell adhesion strength. Although treatment with these inhibitors does not prevent the increase in β -catenin protein levels that occurs after RA treatment, it does prevent the movement of β -catenin to a Triton-insoluble pool at the cell membrane or results in its destabilization there. Similar results were obtained after treatment of cells in low calcium-containing medium to prevent calcium-dependent adhesion. These findings lead us to suggest that the effects of 9-cis-RA on SKBR3 cell β -catenin dynamics are 2-fold. 9-cis-RA increases β -catenin protein stability and, via the action of a serine kinase, mediates its movement to or stabilization at the cell membrane. Although these experiments do not directly address the nature of the kinase(s) involved, retinoids are known to stimulate the activity of PKC via transcriptional and posttranscriptional mechanisms (see Ref. 14 for review). Other studies also implicate PKC activation by O-tetradecanoic 12-phorbol 13-acetate as being a key step in E-cadherin-mediated compaction of mouse embryos (44). Both E-cadherin and β -catenin exist as serine phosphoproteins, and β -catenin coprecipitates with a serine kinase (45) (Lechleider, R., K. Orford, C. L. Sommers, and S. W. Byers, submitted for publication). We also demonstrated that the specific PKC inhibitor bisindolylmaleimide reversed the 9-cis-RA-induced morphological effects. Collectively, these results strongly implicate a serine kinase, perhaps PKC, as being a key mediator of the adhesive response of SKBR3 cells to 9-cis-RA. Although our results only address the mech-

anism by which SKBR3 cell epithelial differentiation is modulated by 9-cis-RA, these cells are also growth inhibited by 9-cis-RA. Whether growth inhibition by 9-cis-RA involves the β -catenin/cadherin system remains to be determined. Other agents that inhibit SKBR3 cell proliferation, such as vitamin D analogs and erb-B2 ligand, do not influence β -catenin protein levels, demonstrating the specificity of retinoid effects and indicating that in these cases, cell growth and changes in β -catenin expression are not necessarily linked. Nevertheless, the known interaction of β -catenin with the tumor suppressor gene APC implies the existence of a retinoid-modulated regulatory network that might integrate contact (adhesion)-dependent signals with those involved in the control of cell proliferation.

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